Novel Autoantibodies in RA and Involvement of SUMO Pathway in the Intrinsic Activation of RASF

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Summary

Rheumatoid arthritis (RA) is, for the time being, an incurable disorder in which the irreversible damage of joints frequently leads to disability. The major pathologic phenomena in RA are the inflammation of the synovial tissue, the autoimmune reaction and synovial hyperplasia resulting in the destruction of cartilage. The autoimmune cells are believed to be central to RA. Moreover, autoantibodies directed at self can be detected even years before the onset of symptoms of the disease. Therefore, autoantibodies are suggested to play a role in the pathogenesis of the disease including its early stages. Moreover, some of them serve as diagnostic markers, disease progression markers, or define subsets of RA patients. Since the identification of novel autoantibodies could be important for understanding specific disease mechanisms, in the first part of the thesis I focused on the characterisation of novel autoantibodies in RA. On the other hand, RA synovial fibroblasts (RASF), which are the resident cells of the hyperplastic synovium in RA, are thought to be effector cells driving the destruction of cartilage in RA. They invade into cartilage and destroy it by releasing considerable quantities of matrix degrading enzymes such as matrix metalloproteinases (MMPs). Moreover, they overexpress small ubiquitin-like modifier (SUMO)-1 and have decreased levels of SUMO specific protease SENP1 which renders them resistant to induced cell death (apoptosis). RASF have recently been shown to have aberrations in the epigenetic code. The epigenetic code is responsible for turning on the genes which are specific for a given cell type (e.g. for fibroblasts of the synovium) and turning off those which should not be active in any particular type of cell. Acetylation of histones is one of the epigenetic modifications responsible for the arrangement of a proper epigenetic code. The field of epigenetics is relatively new and therefore there is not much known about epigenetic modifications in RA. Since histone acetylation modulates gene activity, in the second part of the thesis I hypothesised that the acetylation status could be altered in RASF which might result in the intrinsic overexpression of MMP-1. Furthermore I demonstrated a link between the downregulated SENP1 and the status of histone acetylation in RA.

In the first part of this work, a novel type of autoantibodies has been identified in RA anti-serpin E2 autoantibodies. Serpin E2 antigen was overexpressed in RA compared to OA synovial tissues. Anti-serpin E2 autoantibodies were detected at elevated levels in RA synovial fluids comparing to control patients with osteoarthritis. Most importantly, anti-serpin E2 autoantibodies were shown to have blocking

properties and therefore repressing the function of serpin E2. Since serpin E2 is a natural inhibitor of several serine proteases involved in the pathogenesis of RA, anti-serpin E2 blocking antibodies present in RA could possibly play a functional role in the pathogenesis of the disease by the hyper-activation of urokinase and the plasmin system in RA. However, subsequent studies have shown that these antibodies are not specific for RA and occur also in a certain number of normal appearing individuals.

In the second part of my work it has been demonstrated that in RASF the epigenetic code of MMP-1 was altered. In the promoter of MMP-1 the acetylation of histones was increased, which contributed to the intrinsic overexpression of MMP-1. This was at least partially due to low levels of SENP1 in RASF since the overexpression of SENP1 re-established the proper histone acetylation pattern in the MMP-1 promoter as well as the expression of MMP-1. For this regulation SENP1 required a histone deacetylating enzyme histone deacetylase (HDAC) 4, which decreased the acetylation of histones in the promoter of MMP-1. Most interestingly, this had a functional consequence, since RASF overexpressing SENP1 were significantly less invasive comparing to control RASF.

In summary, there is evidence that anti-serpin E2 autoantibodies are present at increased levels in a certain number of RA patients and block the protective function of serpin E2. To my knowledge they are the first autoantibodies shown to have a distinct function which might be related to the regulation of the proteolytic plasmin system. On the other hand, there is evidence that aberrant expression of SENP1 leads to modifications in the epigenetic code in RASF leading to the overproduction of MMP-1 as well as to an increased invasiveness of the cells. Therefore, re-establishing the expression of SENP1 in RASF could be a promising approach for the therapy in RA.

Zusammenfassung

Die Rheumatoide Arthritis ist eine systemische Auoimmunerkrankung. Hauptcharkteristikum dieser Erkrankung ist die progressive Zerstörung der Gelenke. Meist sind Hand-, Finger, Zehen und Kniegelenke betroffen, in der Regel symmetrisch. Diese Gelenkerkrankung ist bis heute nicht heilbar. Die anhaltende Entzündung der Gelenke resultiert in einer Autoimmunreaktion, bei der Knorpel- und Knochenstrukturen angegriffen und zerstört werden. Mit fortschreitender Erkrankungszeit kommt es zur völligen Zerstörung der betroffenen Gelenke und damit zur Bewegungsunfähigkeit. Die Autoimmunreaktion spielt eine zentrale Rolle in der Pathogenese der Erkrankung. Kürzlich konnte gezeigt werden, dass bereits Jahre vor Ausbruch der Erkrankung Autoantikörper bei diesen Personen nachgewiesen werden können. Autoantikörper spielen auch bei der klinischen Diagnose der Erkrankung eine grosse Rolle. Mittlerweile gibt es bereits Überlegungen wonach die Erkrankung anhand der Autoantikörper-Muster in neue Sub-Gruppen eingeteilt werden könnten. Von der Identifizierung neuer Autoantikörper bei der Rheuamtoiden Artrhitis verspricht man sich die Aufklärung weiterer Pathomechanismen und/oder zusätzliche diagnostische Marker.
Im ersten Teil meiner Arbeit konzentriere ich mich auf die Identifizierung und Charakterisierung neuer, bisher unbekannter Auto-Antikörper in dieser Erkrankung. Dabei untersuchten wir das Auftreten neuer Autoantikörper zum einen systemisch in Seren und zum anderen lokal das Auftreten von Autoantikörper in der Synovialflüssigkeiten betroffener Gelenke von Patienten mit Rheumatoider Arthritis.
Im zweiten Teil meiner Arbeit fokussiere ich mich auf die Bindegewebszelle, den synovialen Fibroblasten (RASF) in den betroffenen Gelenken dieser Patienten. Diese Zellen galten lange Zeit als unbeteiligte Strukturzelle der Bindegewebskapsel. Mittlerweile weiss man, dass diese Zellen die eigentliche Effektorzellen sind, die nach Aktivierung Knorpel- und Knochenzersetzenden Enzyme wie Matrix-Metalloproteinasen (MMPs) freisetzen. Diese als tumorähnliche beschriebene Zellen zeichnen sich unter Anderem durch eine gesteigerte Apoptoseresistenz und damit einer Vermehrung dieser Zellen im betroffenen Gelenk aus. Diese Apoptose-Resistenz wird vermittelt durch eine Überexpression von Ubiquitin-like Modifier (SUMO)-1 einhergehend mit einer verringerten Expression des Gegenspielers der SUMO spezifischen Protease SENP-1. Da Genexpression reguliert werden kann durch epigeneische Modifikationen, haben wir hier untersucht, ob diese Veränderungen zum aktivierten and aggressiven Phänotype dieser Zellen beitragen. Dabei habe

ich mich auf den Einfluss der Acetylierung auf die Expression der Matrixmetalloproteinase 1 (MMP-1) und SENP-1 in synovialen Fibroblasten von Patienten mit Rheumatoider Arthritis konzentriert.
In ersten Teil meiner Arbeit zeige ich dass bei Patienten mit Rheumatoider Arthritis Autoantikörper gegen eine Serin-Protease Serpin-E2 nachweisbar sind. Mittels Immunhistochemie konnten wir das Antigen SerpinE2 in höheren Mengen in der Synovialmembran von Patienten mit RA nachweisen im Vergleich zu Patienten mit Osteoarthritis, einer nicht-entzündlichen degenerativen Erkrankung der Gelenke, die in der Regel zu Vergleichzwecken verwendet wird. In Serum und in Synovialfüssigkeiten fanden wir erhöhte Level von Autoantikörper gegen SerpinE2 in den von uns untersuchen Proben. Neu ist, dass wir das Auftreten dieser Autoantikörper mit einem funktionellen Aspekt in Verbindung bringen konnten. Wir konnten zeigen, dass das Auftreten dieser Autoantikörper, abhängig von der Menge, auch die Funktion der Serinprotease SerpinE2 hemmt. Möglicherweise hemmt das vermehrte Auftreten dieser Autoantikörper bei Patienten mit Rheumatoider Arthritis die Serinprotease SerpinE2 in ihrer Funktion und führt so zu einer Hyperaktivierung derer Zielproteine Urokinase und/oder das Plasmin System. Es muss bemerkt werden, dass spätere Studien gezeigt haben, dass diese Antikörper nicht spezifisch für RA sind und auch bei einer bestimmten Anzahl von gesund erscheinenden Personen vorkommt.
Im zweiten Teil meiner Arbeit konnte ich aufzeigen, dass synovial Fibroblasten von Patienten mit Rheumatoid Arthritis epigenetische Modifikationen bezüglich ihrer Acetlierungsmuster, am Promotor der Gens für MMP-1 aufweisen. Eine Hyperacetylierung des Promotors dieser Matrixmetalloproteinase führt zu deren verstärkten Expression und trägt deshalb zum aktivierten und aggressiven Phänotype der Zellen bei. Eine Aufregulierung von SENP-1 in diesen Zellen reduzierte die Acetylierung durch die Aktivierung von Histondeacetylase 4 (HDAC4) im MMP-1 Promotor und somit dessen Expression. Auch in einem funktionellen in vitro Invasiontest konnten wir zeigen, dass eine Aufregulierung von SENP-1 zu einem signifikant weniger invasiven Phänotyp dieser Zellen führt.
Zusammenfassend konnten wir erhöhte Level von Autoantikörper gegen SerpinE2 bei Patienten mir Rheumatoid Arthritis aufzeigen, die darüber hinaus auch noch eine funktionelle Rolle in der Pathogenese dieser Erkrankung haben, indem sie die Schutzfunktion der Serinprotease SerpinE2 blockieren. Das ist meinem Wissen nach die erste Beschreibung einer Funktion eines Autoantikörpers. Weiterhin konnten wir zeigen, dass die Aufregulierung der Expresssionslevel von SENP-1 den aggressiven und invasiven Phänotyp der synovialen Fibroblasten von Patienten mir Rheumatoider Arthritis aufhebt, da so die apoptoseresistenz dieser Zellen aufgehoben wird und die übermässige Expression von MMP-1 gehemmt werden kann. Dies könnte ein neuer vielversprechender Ansatz für neue Therapien sein.

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Contents

1 Introduction

1.1 Background

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease of which the main characteristic is joint destruction [1]. Since the changes in joints lead to the loss of function, RA frequently results in disability and is accompanied with chronic pain. RA is also the most common inflammatory rheumatic disease and therefore the economical burden is high. Furthermore, even though there has been a major brake through in the treatment of RA in the recent years, the high cost of the treatment as well as a subgroup of non-responders remain an issue. There is, therefore, a priority to identify novel targets for treatment by studying aberrantly inhibited or activated pathways in RA. One of the main features of RA is the presence of high affinity autoantibodies. Certain autoantigens have been implied to contribute to the induction and maintenance of inflammatory reactions against the host tissue(s) [2, 3]. Therefore, the identification of novel autoantigens in RA appears to be very important for a better understanding of specific disease mechanisms. SEREX (Serological Analysis of Recombinant cDNA Expression Libraries), an established tool widely used in screening for autoantigens has been successfully employed to identify autoantibodies associated with different diseases in particular in cancer research [4, 5]. Nevertheless, never before has it been used in the context of RA. In my thesis, using the SEREX technique I identified novel autoantibodies against serine protease inhibitor serpin E2. I could further show that the specific autoantibodies against serpin E2 blocked its inhibitory activity towards serine proteases and therefore they might facilitate the joint destruction in RA. To my knowledge, this study is the first to show a distinct functional property of an autoantibody that might be related to the pathogenesis of RA. On the other hand, more and more evidence suggest the importance of activated RA synovial fibroblasts (RASF) in the pathogenesis of RA. Therefore, studying aberrations in these distinctive cells appears highly promising. Moreover, the growing knowledge in the new field of epigenetics gives us the opportunity to translate it into the context of RASF and the disease itself. The group of Prof. Steffen Gay, which is one of the leading groups in the field of RA in Europe and one of few with a high interest in the novel field of epigenetics of RA, provided therefore the best circumstances for me to perform my PhD. In the second part of the present work I identified a novel aberration in the epigenetic code in RASF involving increased acetylation of histones in the promoter of matrix metalloproteinase (MMP) -1 leading to its overexpression. This phenomenon was linked to a downregulation of SUMO/sentrin specific protease SENP-1. These results provide two distinct pathways as potential targets for the regulation of MMP-1, of which increased expression contributes considerably to the destruction of cartilage in RA.

1.2 Rheumatoid Arthritis

1.2.1 Epidemiology

RA occurs worldwide and affects about 1 percent of the Western Europeans population being the most common of the inflammatory joint diseases. Women have 3 times increased risk of RA compared to men. Urban living individuals have increased risk of RA compared with those living in the rural areas [6]. The disease is heritable to a low extent, since the concordance between the monozygotic twins is 32%. The disease onset is most commonly observed in individuals between 30 and 60 years old.

1.2.2 Clinical aspects

Due to synovitis, the main symptoms of RA are swelling of multiple joints, which become tender, warm and their stiffness cause difficulties to move [7]. Usually, RA affects multiple joints of the hands, feet and cervical spine but also can involve larger joints like the wrist, elbow, shoulder or knee. As the disease progresses, it can also involve other organs such as skin, where the rheumatic nodules can be formed, and lungs. Moreover, in the severe course of RA, vasculitis can also be observed. Patients with RA are more prone to atherosclerosis and have generally an increased risk of cardiovascular events like heart attack and stroke [8]. The extra-articular symptoms occur in about 15% of patients. Other symptoms are the morning stiffness, in or around the joint, which lasts for more then an hour. Radiographic changes can be identified as deterioration of cartilage and erosions of bone. Patients with RA generally have increased levels of rheumatoid factor (RF) and pro-inflammatory cytokines [9, 10]. However, the disease might start long before the onset of symptoms, since increased levels of pro-inflammatory cytokines and some autoantigens might be detected in sera even 10 years before the disease is diagnosed [11, 12, 13, 14]. The patients with RA are classified according to the ACR criteria [15].

1.2.3 Pathophysiology

The pathogenic phenomena in RA occur mainly in the synovial tissues. The main function of the synovial tissue in a healthy joint is supplying synovial fluid which lubricates the cartilage and minimizes the friction of the bones. In RA, the synovial tissue overgrows as a result of infiltration by immune cells and increased survival rate of the resident synovial cells (Figure 1). Activation of the infiltrating immune cells leads to a self-directed attack localized to joints and is followed by joint inflammation (arthritis). Activation of resident synovial cells parallels the inflammation and results in an aggressive invasion of synovial fibroblasts and macrophages into cartilage and bone leading to their damage [16].

RA is characterized by the thickening of the lining layer of the synovium in the affected joints, the infiltration of the synovial tissue with inflammatory cells such as T and B cells and destruction of cartilage and bone by activated RA synovial fibroblasts (RASF) and osteoclasts [17, 18]. Activation of cells of the immune system leads to an increase in inflammation and production of autoantibodies. Activation of RASF leads to an increased production of pro-inflammatory cytokines and chemokines that in turn attract more inflammatory cells to the involved joints [19]. Moreover, RASF excessively express matrix degrading enzymes such as MMPs which leads to irreversible destruction of joint structures [17]. Fur-

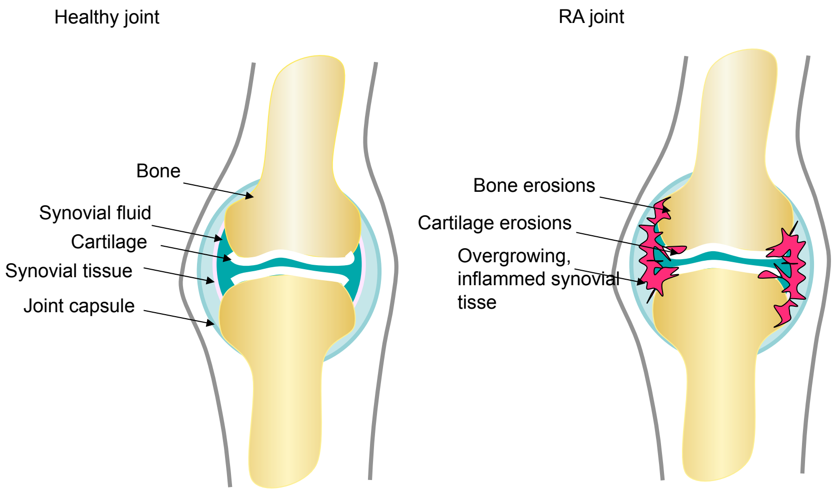

Figure 1.1: Scheme showing a normal joint (A) and RA joint where the hyperplastic synovium invades and damages the cartilage and bone (B).

thermore, RASF have been shown to be resistant to apoptosis, which in turn leads to their accumulation in the synovial tissue and contributes to the activated phenotype of RASF [20]. Therefore, in RA several pathologic phenomena occur simultaneously, namely systemic chronic inflammation, autoimmunity, destruction of cartilage and bone and hyperplasia of the synovium.

1.2.4 Treatment of RA

Currently, a wide range of therapies is available for the treatment of RA. This includes analgesics, such as acetaminophen and opioids, anti-inflammatory drugs, such as glucocorticoids, nonsteroidal anti-inflammatory drugs (NSAIDs) that have both analgesic and anti-inflammatory properties and disease-modifying antirheumatic drugs (DMARDs) which have complex properties [21]. Should these treatments prove ineffective, a new class of drugs, biological-response modifiers so called biologicals are of choice. Biologicals include Rituximab, anti-CD20 antibodies, which is a B cells deprivation agent, tumor necrosis factor α (TNF-α) targeting therapies including Infliximab and Adalimumab, anti-TNF-α antibodies, as well as Etanercept, a soluble receptor for TNF-α, a recombinant inhibitor of interleukin (IL)-1 (Anakinra), a recombinant fusion protein comprising the extracellular domain of human cytotoxic T-lymphocyte antigen (CTLA)-4 and a fragment of the Fc domain of the human immunoglobulin (ig)G1 (Abatacept). Recently, a blocking antibody for IL-6 receptor (Tocilizumab) has been accepted for the treatment of RA. Biologicals are powerful drugs for the majority of patients. However, since not all patients respond to these therapies and there is no cure yet, research has focused on the cells at the site of joint destruction. More attention is now paid, for example, on targeting the bone resorbing osteoclasts [18, 22]. Surprisingly, no interest was raised so far on the notion that, next to the development of autoimmunity and activation of the cytokine driven pathway of joint destruction, a cytokine-independent pathway driven by intrinsically activated RASF is another important part in the pathogenesis of RA [23, 24]. The epigenetic modulations have been proposed to be partially responsible for the intrinsic activation of RASF [25].

1.2.5 Epigenetics and RA

The term epigenetics defines modifications in the genome, inheritable over cell generations but not involving the base pair sequence of the DNA. Epigenetic modulations include DNA methylation and histone modifications such as acetylation, methylation, phosphorylation, ribosylation, ubiquitination and sumoylation (Figure 2).

So far several epigenetic abnormalities have been described in cancer transformed cells. Recent studies on epigenetics, including DNA methylation and modulations of histone acetylation have also contributed to a better understanding of the pathogenesis of RA. The first report on aberrations in the epigenetic code in RA was published already in 1990 [27]. The authors showed that the DNA in T cells of RA

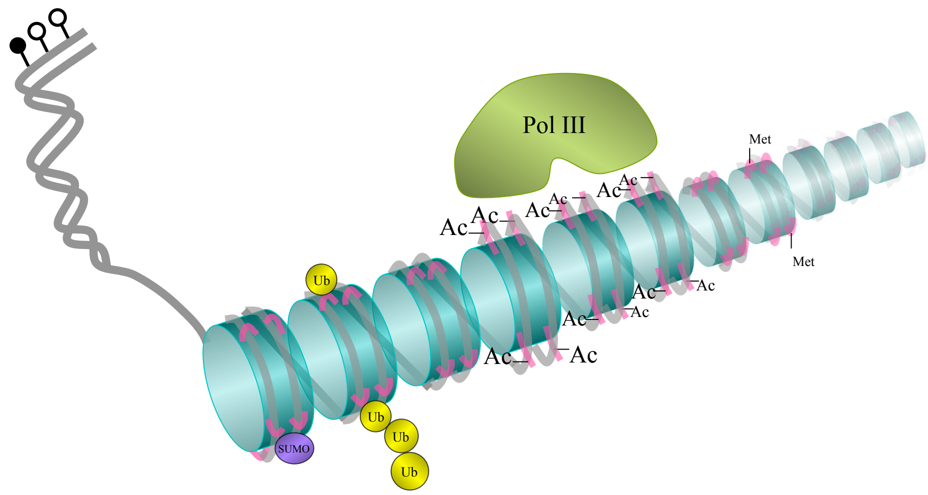

Figure 1.2: Epigenetic modulations of the chromatin. The global hypomethylation of the DNA in RA leads to an increase in gene expression. Hyperacetylation of histones in specific promoter sites renders the DNA region accessible for the transcription machinery. MicroRNA encoded by the DNA silences specific genes in a posttranscriptional manner. Other modifications of histones are indicated, such as sumoylation, ubiquitination, methylation and phosphorylation. Methylated cytosine in DNA is shown as filled and nonmethylated as open circles. The modifications of histone tails are indicated as follows: SUMO sumoylation, Ub ubiquitination, Ac acetylation, P phosphorylation, Me methylation [26].

patients is hypomethylated. Few years later, RASF have been shown to be intrinsically activated and resemble tumor-like cells in some aspects [17]. However, since RASF do not proliferate extensively they are referred to as cells with an activated phenotype [19]. It needs to be shown in the future that the epigenetic changes in the SF could lead to this activated phenotype of RASF.

DNA methylation in RA

DNA methylation in gene promoters containing CpG islands takes place on cytosines of the CpGs, and is connected to a decreased transcription of genes, since transcription factors generally can not bind to promoters containing methylated CpG islands. In this manner genes in heterochromatin are silenced. Recent investigations in our group have shown that RASF have decreased levels of global DNA methylation [25], which leads to an increased expression of cell activating genes and could possibly stimulate the innate immune response via TLR-9. It was reported that the transcription of the retrotransposable element LINE-1 is reactivated in RA synovial tissue due to hypomethylation of CpG islands in its promoter [28]. A single unmethylated CpG in a promoter of IL-6 in monocytes was reported rendering the expression of IL-6 more inducible by LPS stimulation [29]. On the contrary, the CpG island in the promoter of death receptor 3 (DR3), a member of the apoptosis-inducing Fas gene family, was shown to be specifically methylated in synovial cells from patients with RA and the expression of DR3 was accordingly downregulated [30]. The authors concluded that the downregulation of DR3 could play a role in the resistance to apoptosis in RA synovial cells. This finding resembles certain aspects observed in malignant cells where in the stage of global hypomethylation, methylation of specific promoters, for example in tumor suppressor genes occurs.

Histone acetylation in RA

Acetylation of histones H3 and H4 in the nucleosomes of gene promoters is related to the activation of gene expression [31, 32]. This fact is thought to be due to binding of an acetyl group to lysine residue in histones that abolishes the positive charge on the lysine and thus loosens the tight interaction between the positively charged nucleosome and negatively charged DNA. Therefore, the DNA becomes accessible for the transcription machinery. The status of histone acetylation depends on the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). It has been described by Huber et al. that in RA synovial tissues the balance of HAT/HDAC activity is strongly shifted towards histone acetylation [33]. Specifically, a downregulation of HDAC1 and 2 in the synovium of patients with RA has been shown. In other studies, it has been observed that the HDAC inhibitor FK228 inhibits joint swelling, synovial inflammation and joint destruction in mice through the induction of p16INK4a and the upregulation of p21(WAF1/Cip1) [34]. Furthermore FK228 was recently described to suppress the production of VEGF in vivo and to block angiogenesis in synovial tissue in collagen-antibody-induced arthritis [35]. Another HDAC inhibitor, trichostatin A (TSA), was shown to induce cell cycle arrest and apoptosis in RASF and to be particularly effective in combination with ultrasound treatment [36]. TSA was also shown to sensitise RASF to TRAIL-induced apoptosis, however in this study TSA had no effect on apoptosis when used in the absence of TRAIL [37]. The fact that the inhibition of HDACs, which are already less active in RA, is beneficial suggests that HDACs might be misplaced in the subcellular compartments, for example being localised aberrantly to specific promoter sites, and thereby contributing further to the pathogenesis of RA. Therefore, it might be considered whether inhibition or rather redirection to a proper localization of HDACs is feasible for the treatment of RA. In 2006 an interesting study was published comparing gene expression patterns in immortalized B cells from RA patients and from their healthy monozygotic twin siblings [38]. They could compare modulated gene expression in a setting of no genetically diverse background. It needs, however, to be investigated whether some of this modulation could be attributed to epigenetic differences of investigated twins.

MicroRNA in RA

Next to epigenetic modifiers, microRNAs (miRNA) are a recently recognized class of modulators of gene expression [39]. MicroRNA are small non-coding single-stranded RNA molecules that downregulate gene expression on the posttranscriptional level. The expression pattern of miRNA has been suggested to be altered in RA [40]. Indeed, expression of miR-155 and miR-146a was found to be higher in RASF than in osteoarthritis (OA) SF [41, 42]. Moreover, the expression of miR-155 was further enhanced by TNF-α, IL-1β, and the ligands of TLR-2, -3 and -4. Interestingly, the expression of miR-155 in RASF had a repressive effect on the expression of MMP-1 and MMP-3, suggesting an induction of counter regulatory mechanisms in the vicious circle of inflammation and destruction. However, in another study, it has been proposed that miR-146a, even tough expressed at higher levels in RA, is not able to perform its function as a negative regulator for the expression of TRAF-6 and IRAK-1, two molecules involved into the pathway of TNF-α synthesis [43]. Most interestingly, microparticles, which are small vesicles released from activated or dying cells and present abundantly in the synovial fluid of patients with RA [44], also contain miRNA [41, 45]. These observations support the notion that microparticles are intra-cellular mediators of inflammation and entail the activation of synovial cells even more interesting [46].

1.3 Focus of the thesis

The inflammation of the RA synovial tissue, the autoimmune reaction, synovial hyperplasia and the destruction of cartilage are the major pathologic phenomena in RA. In the present work I addressed the following problems: 1) In the aspect of autoimmunity: to identify novel autoantibodies and to study their functional role; 2) In the aspect of cartilage degradation: to study the involvement of the acetylation of histones in the regulation of the expression of matrix degrading enzyme MMP-1. 3) Furthermore I studied the link between the expression of MMP-1 and the SUMOylation pathway, which is known to be responsible for the increased resistance of RASF to apoptosis (Figure 3).

1.3.1 Inflammation/Autoimmunity in RA

Even though it is known that the persistent activation of the immune system in RA leads to autoimmunity [47], the trigger activating the immune response remains unidentified and the pathogenesis of the disease is still not clear. It was suggested that some infectious agents could be cross-reactive with host proteins and by molecular mimicry could trigger the immune response against self epitopes [48]. Another hypothesis grants that the autoimmunity in RA results from pathologic selection of self-reactive cells of the adaptive immune system [49]. The autoreactive T and B cells are though to escape the negative selection and subsequent deletion. Self-reactive leukocytes infiltrate and accumulate in a chronically inflamed RA joint [50]. This is believed to be due to constant delivery of survival

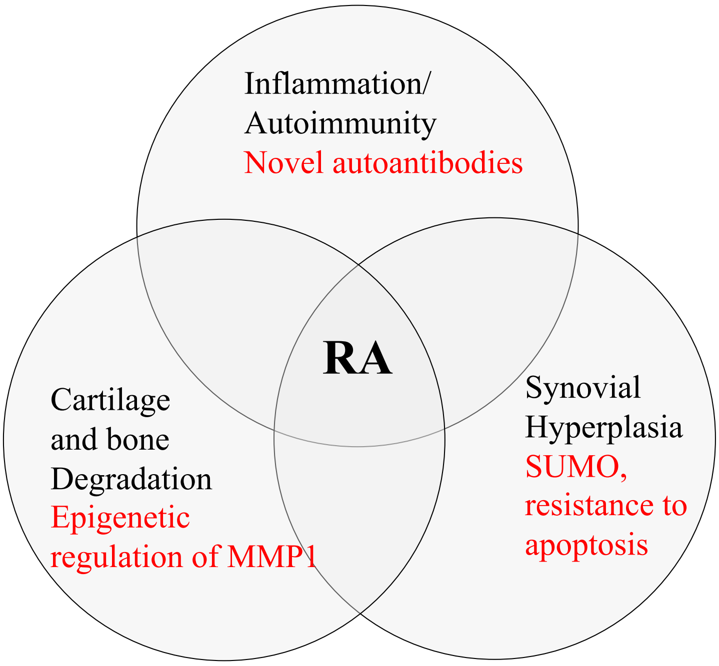

Figure 1.3: Pathologic phenomena in RA. Inflammation, synovial hyperplasia and destruction of cartilage are the major characteristics of RA. The topics addressed in the present thesis are marked in red. Figure modified from [23].

factors by synovial resident cells as well as to a prolonged and disordered resolution phase of inflammatory response. Activated autoreactive B cells produce autoantibodies. The prevalence and a plausible role of autoantibodies in RA is of high interest, since they appear very early in the disease, some even before the onset of symptoms, they partly correlate with the erosions and they can be specific for the disease [12, 14, 51]. Most of the recently developed treatments used in RA target the adaptive immunity. Treatments focusing on B-cells proved to be successful, however only to a limited extent [52, 53, 54]. Targeting CD4 molecule on T-cells was inefficient in clinical trials [55]. Targeting TNF-α, which is thought to stop leukocyte migration and accumulation in the synovium, brought the most effect, however the ACR70 score is reached by only 60% of the treated patients [50, 56, 57].

RA and the innate immune system

The role of the innate immunity is the non-specific recognition of pathogens. The innate immune system constitutes the first defence against microbial particles such as viruses or bacteria, by which it is activated via pathogen associated molecular patterns (PAMPs) ligating to pattern recognition receptors (PRRs). Activation might also occur by other stress factors such as heat, fractures, necrosis, apoptosis, hypoxia or carcinogenesis via diverse molecules expressed on stressed, injured, infected, or transformed human cells that also act as PAMPs [58, 59, 60]. The role of innate immunity in the organism is the identification of foreign substances and their removal by phagocytosis or production of nitric oxide (NO) and the recruitment of immune cells to sites of infection or inflammation by producing cytokines. Moreover, the function of the innate immune system is the activation of complement cascade and of the adaptive immune system by antigen presentation. The cells of the innate immune system are equipped with PRRs to be ready for an immediate response to an offensive event. In this regard, it is interesting that synovial fibroblasts, especially when derived from RA and other inflammatory conditions, express on their surface a range of Toll like receptor molecules (TLRs) belonging to the group of membrane associated PRRs [61, 62]. These cells have therefore the ability to react immediately or within few hours to a wide range of appearing pathogens but also to react to interactions with human cells expressing TLR ligands and induce an immune response. Since RASF express increased levels of TLR2, 3 and 4, they are more prone to activation by diverse TLR ligands when compared to OASF [61, 63] (including endogenous ligands). Thus, it could be hypothesised that RASF function as sensors of danger and can be activated even in the preclinical phase of RA which could lead to the initiation and early perpetuation of the disease. Furthermore, binding of endogenous ligands to TLRs could lead to a constant activation of RASF and maintenance of their pathogenic phenotype.

Adaptive immunity in RA

Infiltration of leukocytes to RA synovium The adaptive immune system composes of lymphocytes - specialized immune cells that recognize specifically a pathogenic epitope and upon this recognition they proliferate proliferate resulting in multiple copies of a single specific cell clone [64]. Then, activated B cells differentiate to plasma cells and produce immunoglobulins (antibodies) binding specifically the corresponding antigen. The antigen-antibody complexes activate the complement system and can be cleared by phagocytosis via Fc receptors expressed on macrophages [65]. T cells specific for the antigen can stimulate a B cell response (T helper; CD4+) or directly kill the pathogen by releasing the cytotoxins perforin and granzymes (T cytotoxic; CD8+) [66]. Whereas helper T 1 (Th1) cells are involved in cellular immunity against intracellular bacteria and viruses by expression of INF-γ and IL-2, Th2 cells mediate the humoral response to parasitic infection and are characterised by the production of IL-4. Th17 cells play a critical role in host defence against extracellular pathogens, particularly those colonizing exposed surfaces like skin, the mucosa of the airways or lining of the intestines. They are the major source of IL-17 and induce the production of chemokine gradient leading to recruitment of the cells of the innate immunity [67]. Another subset of T cells, T regulatory cells (Treg; CD4+. CD25+, Foxp3+) are responsible for limiting the immune response and for immune tolerance to self antigens and therefore they constitute a defense system against self directed attack of the immune system [68]. The effector cytokine of Treg cells is anti-inflammatory IL-10. However, in autoimmune disorders, such as RA, the cells of the adaptive immunity recognize self antigens and propagate a self directed autoimmune reaction. In RA, leukocytes are recruited to the synovial tissue by a sequence of interactions between specific receptors expressed on their surface and correspondent ligands present on the surface of endothelial cells in the area of inflamed synovium or chemokines released from the surrounding cells into the blood stream [69]. Following recruitment to RA synovium, leukocytes find supportive environment for their survival, since activated RASF and macrophages produce anti-apoptotic factors, such as type I interferons [70]. This conditions lead to T-cell survival, where T-cells have high levels of anti-apoptotic Bcl-XL and low levels of pro-apoptotic Bcl-2 [71]. Nevertheless infiltrated T-cells in RA synovium stay in a state of anergy and do not proliferate. Resident cells of the RA synovium actively retain infiltrating immune cells, not only by producing survival factors, but also factors promoting transition of the phenotype of the leukocytes from migratory to stationary. This transition occurs by changing the pattern of adhesion molecules and chemokine receptors expressed on the surface of leukocytes. TGF-β derived from RASF is responsible for up-regulation of CXCR4 expression on synovial T cells [72]. Moreover, the ligand for CXCR4, SDF-1 is produced by synovial endothelial cells and the ligation of SDF-1 promotes adhesion of T cells to adhesion molecules ICAM-1 and fibronectin. Chemokines SDF-1 and BCA-1 which are known to play a role in the lymphoid organogenesis are present at high levels in the RA synovium where they promote B-cell attraction, retention and the formation of germinal centres like structures [73]. These structures are ectopic lymphoid micro-aggregates formed by accumulating lymphocytes [74]. The aggregates are characterized by a secondary lymphoid organ-like architecture, with a central area enrichment of B cells which is surrounded by a T cell peripheral area. Furthermore, these germinal center-like structures have centrally located follicular dendritic cell network and at the edges of the follicles terminally differentiated plasma cells producing autoantibodies. They also develop a specialized vascular apparatus and are characterized by the in situ expression of secondary lymphoid organ chemokines such as CXCL13. While CD4 positive T cells are mostly present in these follicular aggregates, CD8 T cells are found mostly in the synovial fluid. The contact of infiltrating T cells with resident macrophages and RASF in the in the synovial tissue stimulates the two latter to produce TNF-α, IL-1β and other pro-inflammatory cytokines [75]. Thus TNF-α is found at increased levels in RA joint. It was reported that also TNF-α has a role in the establishment of lymphoid tissue [76]. Another factor that promotes the retention of T cells in tissue is recognition of an antigen in general, and in RA synovium, an auto-antigen in particular.

Recognition of auto-antigens In RA, the tolerance of the immune system towards self molecules is broken. It is speculated that infection of susceptible individuals could lead to autoimmunity [77]. Several molecules of viral or bacterial origin were found in RA synovium [78, 79, 80]. These particles are thought to be cross-reactive with endogenous molecules and lead to the deregulation of the immune response. It has been shown that the gp110 protein of Epstein-Barr virus contains the amino-acid sequence QKRAA that is also found in the HLA-DR1 molecule of the host genetically predisposed for RA [81]. Similarly, cross-reactive antibodies against proteus mirabilis proteins, hemolysin and urease, recognise HLA-DR antigen and type XI collagen respectively, and occur in RA sera correlating with the biochemical markers of disease activity [82, 83]. Besides traces of protein, also EBV nucleic acids occur in the synovial fluid of RA patients [84]. The reactivity for self-antigens is also believed to be due to the escape of the auto-reactive cells from the negative selection. Recently, it was reported that the self reactivity of T cells is possibly caused by the low signaling ability of the T cell receptor pathway [85]. In those cells, the response to self-antigen is below the threshold, and as a result they are recognized as tolerant. Autoimmune T cell clones in synovial fluid have been shown to lack the expression or phosphorylation of TCR-ζ, probably due to the interaction with the synovial milieu, and thus have an acquired TCR signaling deficiency [85]. Therefore, upon recognition of the auto-antigen, T cells in RA neither proliferate nor express IL-2 but are rather in quiescent state. Nevertheless, TCR signaling deficient T cells express INF-γ and IL-17 what results in the activation of synovial macrophages, fibroblasts and dendritic cells. Furthermore, they are potent stimulators of antibody production by B cells. Similarly, autoimmune B cells escape counter-selection at tolerance checkpoints both in the bone marrow and in the periphery [86]. Elevation of B lymphocyte stimulator (BLyS), an essential survival factor of B cells belonging to TNF-α cytokine family, in the fluids of RA patients could be responsible for the occurrence of self-reactive B cells [87].

Production of autoantibodies Self reactive T cells release proinflammatory cytokines and promote the production of immunoglobulins directed to self antigens by B cells. Both cytokines and autoantibodies could be detected at increased levels even years before the first symptoms of RA [11, 12, 13, 14]. Although auto-reactive T cells transferred to arthritis-susceptible rat strain could actually transfer the RA-like disease [88], which provided one of the main arguments underscoring the crucial role of T cells in the pathogenesis of inflammatory arthritis, other studies suggest that autoimmune B cells producing autoantibodies play a predominant role in the pathogenesis of RA [89]. There is evidence for deposition of antibodies and complements in the RA synovium [90]. The first described human autoantibodies are rheumatoid factors (RF) being also the most frequently detected autoantibodies in RA patients. RF reacts with the Fc part of immunoglobulins class G, M and A (IgG, IgM and IgA) and IgG RF is considered to be related with more severe inflammation then IgM RF. It has been suggested that the binding of RF to an antibody-antigen complex might be followed by fixation of complement and subsequent attraction of inflammatory cells and their accumulation in the synovium, where they engulf immune complexes and release proteolytic enzymes. Therefore, the involvement of RF may result in inflammatory arthritis. Even though RF are not fully specific for RA, since it is present in 10% of healthy population, several other chronic inflammatory diseases and patients with chronic infection, it has been accepted as one of the American College of Rheumatology (ACR) criteria for RA [91, 92, 93, 94, 95]. In contrast, a remarkable specificity of 98% for RA characterises the antibodies directed to citrullinated peptides (anti-CCP) [96, 97, 98]. Anti-CCP autoantibodies recognize diverse peptides that undergo posttranslational modification of arginine deimination also called citrullination performed by peptidylarginine deiminases (PADI). Among the antigens recognized by anti-CCP antibodies are citrullinated fibrin and citrullinated vimentin [98, 99]. Even though anti-CCPs are present very early in the disease, predict radiographic progression and are associated with HLA-DRB1; they are not yet included in the ACR criteria [100, 101, 102]. Nevertheless, at present, RF concomitantly with anti-CCP show the best predictive value, especially in smokers, over genetic markers for development of RA [103, 104]. Autoantibodies reactive with type II collagen (CII), vimentin, decorin, enolase and aldolase A have also been described in RA [98, 105, 106, 107]. Moreover, several of identified auto-antigens have high homology to antigens from pathogenic organisms, and thus support the hypothesis, that some auto-specificities of immune cells arise from cross-reactivity of the homologous epitope. Most interestingly, autoimmune-prone mice that were lacking the TLR adaptor protein MyD88, had markedly reduced autoantibody titers directed for chromatin, Sm, and rheumatoid factor [108]. Therefore, it is suggested that the innate immunity activation has a promoting effect on the production of autoantibodies by B cells. Reversely, autoantibodies, which recruit complement proteins, can induce innate immunity since complement factors such as C1q are potent inducers of innate immunity response. Furthermore, it has been suggested that both autoantibodies and the initiation of the inflammatory cascade via the alternative complement pathway, which is unrestrained in RA joint, as the cartilage lacks the usual regulatory proteins of the complement system, are needed for the maintenance of the inflammation in RA [90]. Taking into account the frequent presence of the autoantibodies even before the onset of RA symptoms, in the first part of the thesis I focused on the identification and characterisation of novel autoantibodies and their possible functional role in RA.

1.3.2 Destruction of cartilage and bone in RA

Matrix metalloproteinases in RA

The extracellular matrix in a healthy articular cartilage, formed by a highly organised network of different molecules, constitutes a healthy articular cartilage. The proteoglycans, of which aggrecan is predominant, form a polymeric structure forming the protein backbone. They are covalently linked to copolymer chains of negatively charged glycosaminoglycans and non-covalently to hyaluronan. These negatively charged aggregates attract water, swell and therefore provide protection from friction and resiliency. On the other hand, the structural rigidity of the cartilage is conferred by fibers of collagen. Type II collagen is predominant, however types IX and XI also contribute to the formation of fibrils. When type II collagen is destroyed it becomes replaced with type I collagen, which differs in the functional properties. The loss of type II collagen and aggrecan essentially contributes to the progression of joint destruction in RA. Activated RASF from the hyperplastic synovium invade aggressively into the cartilage via breakdown of collagen type II and aggrecans [109, 110, 111]. Isolated RASF sustain their activated phenotype for generations even without any signals from the immune cells. The most striking evidence for the intrinsic activation of RASF comes from the SCID mouse studies [17, 112]. In the SCID mouse model of RA, normal human cartilage and RASF are co-implanted on the sponge under the renal capsule of immunodeficient mice (Figure 4). It has been shown that 60 days after co-implantation, RASF aggressively invaded into the cartilage [17].

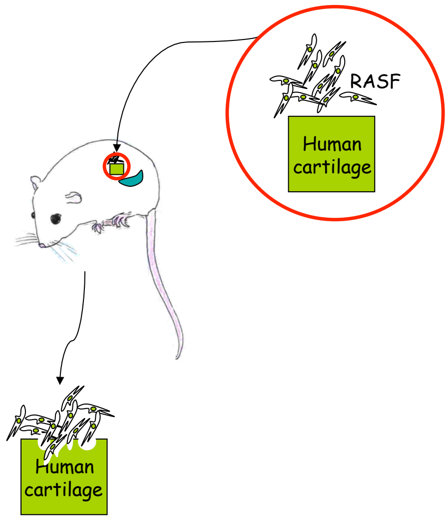

Figure 1.4: SCID mouse model of RA. Normal human cartilage and RASF are coimplanted to immunodeficient SCID mouse into the fat tissue under the renal capsule. 60 days after implantation mouse is sacrificed and the human cartilage with invading RASF analysed.

The molecular basis of RASF activation is characterized by an abnormal expression of protooncogenes and an induction of matrix-degrading enzymes such as MMPs and cathepsins [113, 114]. Therefore, RASF are referred to as cells with an activated phenotype. MMPs are involved in the tissue degradation, since they have proteolytic activity towards components of the extracellular matrix. Moreover, they were described to play a role in the cleavage of cell surface receptors, the release of FasL and activation or deactivation of chemokines [115, 116]. MMPs have been divided into 5 groups according to their substrate specificities. Collagenases (MMP-1, -8, -13) target collagens, the galetinases (MMP-2 and -9) degrade type

IV collagen in the basement membrane, stromelysins (MMP-3, -10 and -11) have non-collagen matrix proteins for substrates. Two other groups are membrane-type MMPs (MMP-14, -15, -16, -17, -24, -25) and a diverse subgroup of MMP-7, -11, -12, -20 and -23. Two collagenases, MMP-1 and -13 are rate limiting in the collagen degradation process and thus are believed to have a key role in the pathogenesis of RA [117, 118]. Moreover, MMP-13 has also activity towards aggrecan. The majority of MMP-1 is of synovial cell origin, while MMP-13 is produced mainly by chondrocytes in the arthritic cartilage. Moreover, MMP-2, -3 and -9, that have proteolitic specificities also against non-collagen matrix components are also found at increased levels in RA joints [117, 118]. The release of fragments of cleaved matrix components into the circulation is believed to stimulate an autoimmune response in the susceptible individuals [119]. Even though the expression of MMPs is induced by TNF-α and IL-1β, the invasive RASF have been shown to have increased basic levels of MMPs as shown in the SCID mouse model and in vitro cultures of RASF. MMPs have natural inhibitors, tissue inhibitor of matrix metalloproteinase (TIMP) that are elevated in RA joint. The levels of TIMPs are however lower than those of MMPs and therefore, TIMPs are not sufficiently effective [120]. In the SCID mouse model of RA the overexpression of TIMP-1 and TIMP-3 in RASF resulted in a reduction of their invasiveness [121]. Paradoxically, overexpression of TIMPs seemed to increase cell growth and invasion in collagen induced arthritis model [122]. There have been attempts to design effective synthetic inhibitors of MMPs that could be used as a treatment for RA, unfortunately none has proved successful. Therefore, studying the cellular and molecular mechanisms that lead to the increased production of MMPs in RA might provide targets for therapy. The promoters of MMP-1, -3, -9 and -13 contain binding sites for AP-1, which is located in the proximity of transcription starting point and is essential for the gene expression [123]. AP1 can interact with other transcription factors, like Ets that binds to polyoma virus enhancer activator-3 (PEA-3/ETS) sites. Furthermore, MMP-1 promoter contains another AP-1 binding site at -1602 base pairs upstream from the transcription starting point. IL-1 and TNF-α stimulate expression of MMPs via MAPK pathway leading to binding of AP-1 and Ets to their binding sites in the promoters of MMPs and via targeting of another transcriptional activator, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) to the promoters of MMP-1, -3 and -9. Even though the promoter of MMP-13 does not contain the binding site for NFκB, inhibiting NFκB decreases the induction of MMP-13 expression in stimulated cells. Transforming growth factor β (TGF-β) is another regulator of expression of MMPs which can have both stimulatory and repressive effects [124]. Moreover, it has been described that the expression of MMP-1 and MMP-13 is regulated by epigenetic modulations [124, 125]. Since there is accumulating evidence for the aberrations in the epigenetic code in RA, in the second part of my thesis I focused on the characterisation of histone acetylation in the promoter of MMP-1.

Serine proteases in RA

Several members of another class of proteases, serine proteases, were reported to contribute to the pathogenesis of RA [126, 127, 128, 129, 130]. Serine proteases such as urokinase-type plasminogen activator (uPA), plasmin, tissue plasminogen activator (tPA), thrombin and trypsin have increased activity in RA. Increased expression and activity of uPA, which is an enzyme responsible for processing plasminogen into plasmin, was observed in the lining layer of RA synovial tissues when compared to synovial tissues from OA patients or healthy individuals [130]. Also the expression of two other molecules involved in the uPA signalling belonging to the cell-associated fibrinolytic system, the specific uPA receptor (uPAR) and the plasminogen activator inhibitor-type 1 (PAI-1), were reported to be increased in the synovial tissues of RA joints and in RASF [130, 131]. Interestingly, upregulated fibrinolitic system was found to contribute to the spontaneous and uPA-challenged invasion and proliferation of RASF [131]. In addition, deficiency of the zymogen of plasmin, plasminogen, was described to be protective in the collagen induced arthritis (CIA) mouse model of RA [132]. Similarly, mice deficient in uPA had decreased incidence and severity of CIA. Even though the development of the autoantibodies was not different compared to the wild type mice, the infiltration of inflammatory cells into synovial tissues as well as inflammation reaction were impaired in the plasminogen activator system deficient mice. Therefore, plasmin is thought to be a key player in the early phase of pathologic changes of CIA in mice and possibly of RA in human. Thrombin-like activity was reported to be significantly higher in RA than in OA synovial fluids and correlated with cathepsin B activity [129]. Furthermore, thrombin enhanced the proliferation of synovial fluid cells as well as the release of IL-8 by them. Thus, thrombin was concluded to be involved in promoting inflammation through the production of IL-8 and the recruitment of leukocytes, which release cathepsin B into the synovial fluid. Another serine protease, trypsin-2 of human origin was shown to be capable of cleaving collagen type II and was detected in the RA synovial tissue as well as in the synovial fluid of affected joints [127]. Therefore, it was proposed to be a possible contributor to the pathogenesis of RA via degradation of collagen type II. Serine proteases cleave their substrates in a two-step process. First the catalytic serine by a nucleophilic attack on the peptide bond of the substrate causes release of a new N-terminus and forms a covalent bond with the substrate. Next, the bond between the protease and the substrate is hydrolysed and the protease released [133]. Serine proteases have their natural inhibitors which constitute a protein family of serpins (serine protease inhibitors). Serpins are the largest and most diverse family of protease inhibitors [134] containing members in higher animals, nematodes, insects, plants, bacteria, archea and viruses [135, 136]. The members of the serpin family are classified into 16 groups according to the filogenetic analysis. The majority of serpin family members have inhibitory activity towards serine proteases and they share the mechanism of inhibition. They typically have three β-sheets and eight or nine α-helices. They also contain an exposed reactive centre loop (RCL) in the C- terminal part [137]. RCL determines target specificity and is responsible for the interaction with serine proteases forming a bait. If RCL is not cleaved, serpin is in a stressed conformation. When the serine protease cleaves RCL it forms a covalent bond with the serpin and causes its transition to the relaxed conformation [138, 139]. During the transition RCL fits to one of the β-sheets of serpin carrying conjugated protease to the opposite site of the serpin molecule also causing a change in its conformation. In this new conformation the serine protease hydrolyses the covalent bond with the serpin extremely slowly [140]. In the meanwhile the complex serine protease-serpin is internalised to the cell where it undergoes proteotolytic degradation. The majority of serpins function extracellularly, they play a role in regulation of the proteolytic cascades in blood clotting (e.g. antithrombin), the inflammatory response (e.g.

antitrypsin, antichymotrypsin) and tissue remodelling (e.g. PAI-1). Deficiency of serpins in humans results in diverse pathological states such as emphysema, cirrhosis, thrombosis, dementia, cardiovascular disease and tumor progression [141, 142]. Since one of the serpins serpin E2 was identified in this work as an autoantigen in RA, I further characterised the distribution of specific anti-serpin E2 antibodies and whether the binding of specific autoantibodies influences the inhibitory activity of serpin E2.

Osteoclastogenesis

Destruction of the cartilage in RA is accompanied by the activation of pathways that result in bone degradation. Osteoclasts are the cells responsible for dissolving bone structures. An increased formation of osteoclasts in the synovium is one of characteristic features in the pathogenesis of RA. Monocyte precursor cells express on their surface the receptor activator of NF-κB (RANK) belonging to the TNF receptor family. Stimulation of these cells by the RANK ligand (RANKL) leads to their maturation into osteoclasts in a process of osteoclastogenesis. RANKL was found to be present at sites of bone erosion in RA and is known to be produced by RASF [143]. TNF-α and IL-1β induce osteoclastogenesis via upregulation of bone morphogenetic protein-2 and -6 in RASF [144]. Since RASF have the potential to drive cartilage and bone degradation by producing MMPs and stimulating osteoclastogenesis, it is therefore suggested that they could be driving the destruction of the joints in RA.

1.3.3 Hyperplasia of synovium

In a normal joint, the lining layer of the synovium composed of synovial fibroblasts and macrophages is only around two cells thick. In RA, however, synovial lining consists of up to 8 cells in depth (Figure 5). Also the sublining layer of the synovial tissue which is built by the connective tissue is characterised by increased cellularity. In RA, the normally flat surface of the synovium is highly folded and therefore the synovial surface is increased by even two orders of magnitude. It has been reported that synovial hyperplasia correlates with joint erosions in RA [145, 146]. Surprisingly, the levels of cell division in hyperplastic synovium do not appear to be significantly increased [20]. Hyperplasia can be partly attributed to the active retention and promotion of survival of infiltrating immune cells by the resident stromal cells as discussed beforehand. Nevertheless, the increased numbers of resident RASF and macrophages also contribute significantly to the formation of the hyperplastic synovium. It remains a question of debate whether RASF have increased levels of proliferation. Qu et al. determined the that the expression of markers of proliferation such as proliferating cell nuclear antigen (PCNA), c-myc, and the nucleolar organizer regions (NOR) are increased in RASF of the synovial lining layer [147], however this result was not confirmed by other studies where the expression of Ki-67, the mitosis rate, and the 3H-thymidine uptake, as well as PCNA and NOR showed no difference between RASF and control cells [148, 149, 150]. In contrast, it is generally accepted that RASF are resistant to apoptosis and this accounts at least partially for their increased accumulation in the synovium.

Resistance to apoptosis

Increased resistance to induced apoptosis is one of the most characteristic features of RASF. RASF were shown to be resistant to apoptosis induced by TRAIL, FasL and TNF-α [37, 151, 152]. The respective receptors, TRAIL receptor, Fas and TNF receptor I (TNFRI) contain the death domain, which, upon binding of a corresponding ligand to the receptor, is bound by Fas-associated protein with the Death Domain (FADD). FADD is an adaptor molecule, which recruits caspase-8 to the receptor resulting in the formation of the death inducing signaling complex (DISC), activation of cysteine protease cascade and ultimately in apoptosis. Despite expressing all three types of receptors, RASF are resistant to Fas, TRAIL and TNF-α mediated cell death. Furthermore, the resistance of RASF to apoptosis can not be explained by the absence of apoptosis inducing ligands since soluble TRAIL, FasL and TNF-α were found at increased levels in synovial fluids of patients with RA compared with OA. Interestingly, it has been shown that RASF produce increased levels of the anti-apoptotic molecule small ubiquitin-like modifier (SUMO) -1/sentrin which is known to bind to the cytoplasmic domains of Fas and TNFRI and thereby to protect cells from both FasL and TNF-induced cell death. Franz et

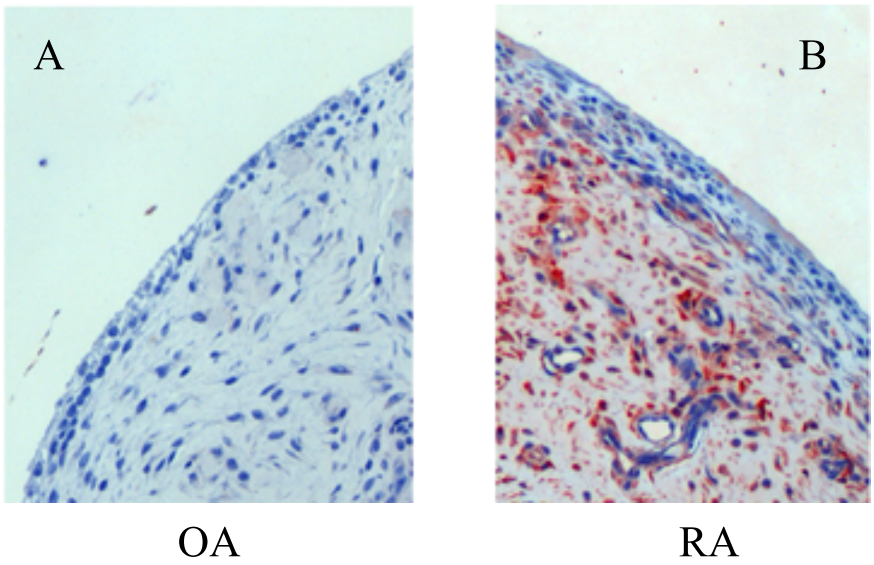

Figure 1.5: Hyperplastic synovium in RA has increased thickness of intimal lining layer. In OA the lining layer is two cells thick (A) and in RA it has 6-8 cells in depth (B).

al. has shown that SUMO-1 was overexpressed in RASF aggressively invading into cartilage in patients as well as in the SCID mouse model [153]. However, SUMO-1 could not be shown to be localised in the cytoplasm in RASF, but was rather present in the nucleus, mainly in the subnuclear structures called promyelocytic leukemia (PML) nuclear bodies [154]. Therefore, SUMO-1 failed to interact with the death domains of Fas and TNFRI in RASF. Nevertheless, in the absence of SUMO-1, RASF were prone to FasL induced apoptosis. Further investigations of the role of SUMO-1 in RASF have demonstrated that the intrinsic overexpression of SUMO-1 in RASF leads to the accumulation of the pro-apoptotic DAXX in the PML nuclear bodies. DAXX is therefore retained in the PML and can not exhibit its pro-apoptotic functions, which contributes to the resistance of RASF to Fas induced apoptosis [154]. Moreover, SUMO specific protease SENP1 was shown to be downregulated in RASF and its re-expression leads to an increased apoptotic response induced by FasL.

SUMO

SUMO-1 together with SUMO-2, -3 and -4 belong to the ubiquitin-like protein family [155] and they modulate proteins by conjugating to them in a reaction of sumoylation. Sumoylation, analogously to ubiquitination, is a reversible post-translational modification. It is a multi-step reaction initiated by a cleavage of a precursor form of SUMO to a mature protein by a sentrin specific protease (SENP) [156, 157]. Three enzymes take part in the covalent attachment of SUMO to the substrates; E1 activating enzyme, E2 conjugating enzyme and E3 - a SUMO ligase [158, 159]. Reversely, SENPs deconjugate SUMO from substrates by cleaving the isopeptide bond [156, 157]. Up to date there are six SENP homologues identified in humans (SENP1, 2, 3, 5, 6 and 7) [160]. They differ in the localization and specificity towards different isotypes of SUMO. SENP1 is a nuclear protein mainly localized in the PML nuclear bodies. It has the highest specificity towards SUMO-1, but also can cleave SUMO-2 and -3 [161, 162]. SUMO-1, when bound to substrates, can influence their properties such as stability, localization, interaction with other proteins and activity [156, 163, 164]. A range of transcription factors including c-Jun, Ets1 and C/EBP have been reported to be substrates for sumoylation. The majority of transcriptional activators, when SUMOylated, show decreased activity in inducing transcription, however some transcription activators such as Ikaros, APA-1, CREB, ER, FAT1, HIF1a, HSF, MITF, P45/NF-E2, p53, and Tcf-4 have, on the contrary, higher activity in the SUMOylated form [165]. Similarly, a range of transcription inhibitors are known to be SUMO modified. PML, DAXX, histone deacetylase 1 and 4 (HDAC1 and 4) are known to be post-translationally modified by SUMO-1 and this modification is suggested to increase their activity as negative regulators of transcription [166, 167, 168, 169]. However, the reports on the influence of sumoylation on the activity of HDAC4 are not conclusive. While a sumoylation-deficient mutant of HDAC4 has a slightly decreased ability to repress the transcription, the nuclear transport of HDAC4 depends on the modification by SUMO-1. This implies that sumoylation of HDAC4 rather contributes to the change in the localisation of HDAC4 than influence the activity directly. HDAC4 was reported to co-localise with PML in the nuclear bodies in neurons in neuronal intranuclear inclusion disease [170, 171]. Gao et al observed that HDAC4, 5 and 7 are localised to PML nuclear bodies and the localisation of HDAC7 into PML promoted the transcription of MMP-10 [172, 173]. Since it is known that the levels of SUMO-1 are increased while SENP1 is decreased in RASF I studied whether the re-expression of SENP1 could regulate the localisation of HDAC4 to the specific promoter sites leading to the regulation of gene expression. I focused on the regulation of MMP-1 since it is overexpressed by RASF and is rate limiting for the cartilage destruction in RA.

1.4 Objective

1.4.1 Rationale

Different cell types such as T cells, B cells, macrophages and synovial fibroblasts are involved in the pathogenesis of RA. Therefore, the intertwining adaptive and innate immune responses have been proposed to have a crucial role in the disease. Since RA is an autoimmune disorder, B cells producing autoantibodies have been long known to be involved into perpetuation of RA. Nevertheless only since few years it is known that autoantibodies can appear in the sera of healthy individuals, who, few years later, develop RA. Therefore, the identification of autoantibodies and studying their role in RA is an exciting and challenging task. It could lead to a better understanding of the disease processes and perhaps will help in the identification of the trigger of RA. While B cells and the adaptive immune system have long been recognised to be essential players in the development of RA, only recently it has been agreed that RASF have a main role in the perpetuation of RA in the absence of the adaptive immune system. There is evidence that RASF play a role in the innate immunity since they express a range of TLR. Moreover, since RASF are intrinsically activated, they aggressively invade cartilage as well as have increased survival levels. The intrinsic overexpression of an anti-apoptotic molecule SUMO-1 in RASF renders resistance to apoptosis after stimulation with apoptosis inducer FasL. It has been shown that the resistance to apoptosis can be broken in RASF by decreasing levels of sumoylation. Even though SUMO-1 is a modulator of many proteins including transcription factors and epigenetic modulators, it has never been investigated whether the characteristic overexpression of SUMO-1 in RASF could promote changes in gene expression pattern and result in the aggressive phenotype of cells. We hypothesized that the increased levels of SUMO-1 modified proteins in RASF could lead to an increase in the production of MMPs and therefore promote the aggressive behaviour of cells.

1.4.2 Aims

A subset of B cells in RA shows autoimmune reactivity and, as a result, produces autoantibodies. The autoantibodies have been suggested to play a role in triggering and maintenance of RA, however, neither of these phenomena has been assigned to a particular known autoantibody species. Therefore in Chapter 2 I report the work focusing on the identification of novel autoantibodies in RA. Since we identified autoantibodies directed against serpin E2, a known inhibitor of serine proteases, we investigated the influence of the interaction of serpin E2 with serpin E2 directed autoantibodies on the function of serpin E2. Synovial fibroblasts play a key role in the joint destruction in RA and produce large amounts of matrix degrading enzymes and cytokines. The intrinsic activation of RASF might be a result of overexpression of SUMO-1 as well as epigenetic changes influencing gene expression pattern of MMPs and cytokines. Previous studies in our group have shown that the intrinsic overexpression of SUMO-1 in RASF leads to the resistance of the cells to induced cell death. Even though it is known that sumoylation can influence gene expression and the overexpression of SUMO-1 paralleled by a decrease in SENP1 levels is characteristic for RASF, it has never been studied whether this could contribute to the aggressive phenotype of RASF by influencing the expression of MMPs. In Chapter 3 I assessed the levels of expression of MMP-1 in RASF transfected with SUMO specific protease SENP1 in comparison to mock transfected cells. Since the levels of MMPs were downregulated in the cells overexpressing SENP1 we further studied the mechanism of this downregulation. In summary, showing the functional role of aberrations in the sumoylation pathway as well as epigenetic changes that could be partially due to the modified levels of SUMO-1 and SENP1 in RASF, additional substantial prove is provided to the body of evidence for the intrinsic activation of RASF.

2 Functional autoantibodies against serpin E2 in rheumatoid arthritis

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Functional Autoantibodies Against Serpin E2 in Rheumatoid Arthritis

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Objective. **To search for novel autoantibodies in patients with rheumatoid arthritis (RA) in an effort to better understand the processes of joint destruction in this disease.**

Methods. **Using a modified SEREX technique and complementary DNA derived from RA synovium, serpin E2 was identified as a novel autoantigen and was analyzed by immunohistochemistry. Levels of anti–serpin E2 autoantibodies in serum and synovial fluid from patients with RA, osteoarthritis (OA), psoriatic arthritis, and ankylosing spondylitis, and/or from healthy individuals were assessed by enzyme-linked immunosorbent assay. Since serpin E2 is an inhibitor of serine proteases, we studied the inhibitory activity of serpin E2 toward its target, urokinase plasminogen activator (uPA), in vitro in the presence of isolated anti–serpin E2 autoantibodies and in vivo using the uPA activity assay.**

Results. **We identified autoantibodies against serpin E2 by the SEREX technique. Serpin E2 was overexpressed in RA synovial tissues as compared with OA synovial tissues. Significantly higher levels of anti–serpin E2 autoantibodies were present in samples of synovial fluid (28%) and serum (22%) from RA patients as compared with OA patients (0 and 6%, respectively) or with healthy individuals (6% of sera). Most importantly, anti–serpin E2 autoantibodies isolated from RA sera reversed the inhibitory activity of serpin E2 by 70%. Furthermore, the levels of anti–serpin E2 autoantibodies correlated with the uPA activity in vivo.**

Conclusion. **This study characterizes a functional property of a novel autoantibody in RA. Since anti–serpin E2 autoantibodies interfere with the inhibitory activity of serpin E2 toward serine proteases, they might facilitate the joint destruction in RA.**

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint destruction. While it is known that persistent activation of the immune system in RA leads to autoimmunity, the trigger that activates the immune response remains unclear. Nevertheless, increased levels of cytokines and autoantibodies may be detectable years before the first symptoms of RA (1–4). In 1992, Silman et al (1) reported the identification of autoantibodies in serum samples from healthy subjects as many as 10 years before they developed RA.

The autoantibodies most frequently detected in RA patients are rheumatoid factor (RF) and antibodies directed toward citrullinated peptides (anti-CCP). Even though RF is not specific for RA, since it is present in 10% of the healthy population, in patients with several

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other chronic inflammatory diseases, and in those with chronic infection, it has been accepted as one of the American College of Rheumatology (ACR) criteria for RA (5–8). In contrast, anti-CCP antibodies have a remarkable 98% specificity for RA (9–11). Even though anti-CCP antibodies are present very early in the disease, predict radiographic progression, and are associated with HLA–DRB1, they are not yet included in the ACR criteria for RA (12–14). Citrullinated fibrin and citrullinated vimentin are among the antigens recognized by anti-CCP antibodies (11,15). Several other autoantibodies have been described thus far in RA, including autoantibodies reactive with type II collagen, decorin, enolase, and aldolase A (16–19).

Antibody-producing plasma cells and B cells are key players in the inflammatory process of the disease, and depletion of B cells using anti-CD20 antibodies has been approved for the treatment of RA (20). Nonetheless, it is still not clear how they contribute to the pathogenesis of RA. The identification of autoantibodies and the definition of their possible pathologic role are therefore a challenging task.

Serologic analysis of a recombinant human complementary DNA (cDNA) expression library (SEREX) is a valuable screening method used extensively for the identification of autoantibodies related to different diseases (21–26). The SEREX technique allows for the fast identification of all cDNA-encoded proteins in a given tissue sample that are recognized by autologous sera. The main advantage of SEREX over classic methods such as Western blotting is the possibility of identifying a broad spectrum of autoantibodies that are reactive with proteins expressed extracellularly and intracellularly, since it involves the screening of a complete gene expression library.

In the present study, we used a modified SEREX technique and a cDNA library obtained from RA synovial tissue as a screening method to identify novel targets for autoantibodies in RA. We found increased levels of autoantibodies to serpin E2 in synovial fluid and serum from patients with RA. In search of a functional role of these autoantibodies, we found that anti–serpin E2 autoantibodies interfered with the inhibitory activity of serpin E2 and, thus, might contribute to the destructive process in the joints of RA patients.

PATIENTS AND METHODS

Synovial fluid and serum samples. Synovial fluid samples were obtained from 44 patients with RA, 18 with osteoarthritis (OA), 10 with psoriatic arthritis (PsA), and 10 with ankylosing spondylitis (AS) and were stored at –80°C. Before analysis, synovial fluid samples were treated with hyaluronidase (1 mg/ml for 1 hour at room temperature). Serum samples were obtained from 183 RA patients (134 women and 49 men with a mean age of 46 years [range 13–75 years]; RF negative in 37), 64 healthy subjects matched for sex and age with the RA patients (41 women and 23 men with a mean age of 45 years [range 24–75 years]), 34 OA patients (20 women and 14 men with a mean age of 65.5 years [range 40–83 years]), 11 PsA patients (4 women and 7 men with a mean age of 44 years [range 25–52 years]), and 14 AS patients (5 women and 9 men with a mean age of 41 years [range 26–52 years]). Serum samples were stored at –80°C until analyzed.

The synovial fluid and serum samples were obtained from clinics in Zurich, Switzerland, Manchester, UK, and Erlangen, Germany. All RA patients fulfilled the ACR (formerly, the American Rheumatism Association) criteria (27). All experiments were performed after obtaining informed consent from the study participants.

Detection of autoantibodies in RA synovial fluid by SEREX. We used a modified SEREX technique in which synovial fluid was included for the identification of novel autoantibodies in RA patients. A human RA synoviocyte λ cDNA library kit obtained from Stratagene (Cedar Creek, TX) was used for these analyses according to the manufacturer's instructions and as previously described (21–25). XL1-Blue MRF′ cells were transfected with a premade λ phage RA synovium cDNA library, and the expression of recombinant proteins was induced with IPTG. Recombinant proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Synovial fluid from a patient with RA was preabsorbed against bacterial and λ phage proteins prior to analysis. Membranes were blocked with 5% milk in Tris buffered saline (TBS; 20 m*M* Tris HCl, 150 m*M* NaCl) and incubated overnight at 4°C with synovial fluid diluted 1:200. Reactive antibodies were detected with goat anti-human IgG alkaline phosphatase–conjugated antibodies (Sigma-Aldrich, Basel, Switzerland) and visualized with nitroblue tetrazolium/BCIP (Roche, Reinach, Switzerland). The positive clones were confirmed by a second round of screening, and plaques were isolated. For excision of the plasmids, the SOLR bacterial strain was infected with the isolated phagemid. In vitro excision of the plasmid DNA was performed using the ExAssist helper phage provided in the cDNA library kit. DNA was isolated and sequenced using T7 primers (Microsynth, Balgach, Switzerland).

Identification of the sequences of the detected autoantigens was performed using the National Center for Biotechnology Information gene bank (online at http://www.ncbi.nlm.nih.gov) by sequence alignment in BLAST.

Immunohistochemistry for serpin E2 in RA and OA synovial tissues. Synovial tissues were obtained from RA (n = 10) and OA (n = 10) patients undergoing joint replacement surgery. Tissue samples were immediately fixed in paraformaldehyde and embedded in paraffin. Subsequently, 3-μm sections were prepared. Following deparaffinization, nonspecific binding was blocked by incubation for 1 hour in a blocking solution (4% nonfat dry milk and 2% goat serum in TBS, pH 7.4). Slides were then incubated for 1 hour with mouse anti-human serpin E2 IgG antibodies (a generous gift of Marie-Christine Bouton, University Paris 7, Paris, France) at a concentration of 14 μg/ml, followed by incubation with alkaline phosphatase–conjugated goat anti-mouse anti-immunoglobulins in phosphate buffered saline (Dako, Baar, Switzerland). The antigen–antibody reaction was visualized using Fast Red substrate (Dako). In control experiments, primary antibodies were replaced by a mouse IgG isotype at the same concentration. The slides were counterstained with hematoxylin for 1 minute, dehydrated, and mounted. All steps were performed at room temperature. A grading system of 0–3 was used to score the expression levels of serpin E2 in synovial tissues, where 0 = no expression, 1 = weak expression, 2 = medium strong expression, and 3 = strong expression.

Enzyme-linked immunosorbent assay (ELISA) for anti–serpin E2 autoantibodies in synovial fluids and sera. To establish an ELISA, conditions were optimized by using various concentrations (10–100 ng/ml) of recombinant human serpin E2 (R&D Systems, Abingdon, UK), a range of dilutions (1:10–1:1000) of hyaluronidase-treated synovial fluid or sera, and different concentrations (90–900 ng/ml) of horseradish peroxidase (HRP)–conjugated goat anti-human IgG antibodies (Jackson ImmunoResearch, Magden, Switzerland). The following conditions were used for the analysis: plates were coated with 100 ng/ml of recombinant human serpin E2 in a coating buffer (0.53% Na_2CO_3, 0.42% $NaHCO_3$, 0.1% sodium azide, pH 9.6) and incubated overnight at 4°C. Nonspecific immune interactions were blocked with 3% bovine serum albumin (BSA) in TBS containing 0.05% Tween 20 (TBST). This was followed by incubation with diluted synovial fluid samples (1:200 in TBST containing 1% BSA) or sera (1:500 in TBST containing 1% BSA) in duplicate for 2 hours at room temperature. HRP-conjugated goat anti-human IgG antibodies were added (90 ng/ml in TBST containing 1% BSA) for 1 hour.

The reaction was visualized using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (BD Biosciences, Allschwil, Switzerland), and the reaction was stopped with 10% orthophosphoric acid (Fluka, Buchs, Switzerland). As a positive control mouse anti-human serpin E2 IgG antibodies (a generous gift of Marie-Christine Bouton, University Paris 7, Paris, France) detected by HRP-conjugated rabbit anti-mouse antibodies (Dako) and visualized using TMB were used.

Plates were read at a wavelength of 450 nm. One selected RA serum was used for preparation of the standard curve and for normalization of the results of all of the tests that were performed (set at 100 arbitrary units [AU]). Cutoff values were calculated as mean + 2 SD of the value in OA patients for synovial fluid and as the mean + 2 SD of the value in healthy individuals for serum. Values below the respective cutoff levels were considered to be in the normal range.

Isolation of autoantibodies specific for serpin E2 from sera. Serpin E2–specific autoantibodies were isolated from sera as previously described (28). Briefly, 4 ml of CNBr-activated Sepharose 4 Fast Flow (GE Healthcare, Otelfingen, Switzerland) was washed 12 times with 4 ml of cold 1 m*M* HCl, pH 3, and after each washing was incubated at 4°C for 5 minutes. Conjugation of recombinant human serpin E2 or the irrelevant autoantigen pituitary tumor–transforming gene 1–interacting protein (PTTG-1IP) (2.5 μg in 2.5 ml of 0.1*M* $NaHCO_3$, 0.5*M* NaCl, pH 8.3, buffer) to packed CNBr-activated Sepharose 4 Fast Flow was performed at pH 7.5 for 2 hours at 4°C, followed by 3 hours of incubation at room temperature. The Sepharose gel was then washed with buffer (0.1*M* $NaHCO_3$, 0.5*M* NaCl, pH 8.3) and blocked with a 2.5× excess of 0.1*M* Tris HCl, pH 8, for 2 hours at room temperature. The Sepharose gel was split into 1-ml portions and washed alternately (6 times) with 3 ml of 0.1 acetate buffer, pH 3.4, containing 0.5*M* NaCl and 0.1*M* Tris HCl buffer, pH 8.0. The gel portions were washed once with PBS and used immediately for the isolation of antibodies from sera or were stored at 4°C.

The capture of autoantibodies against serpin E2 was performed by incubation of 1 ml of human serum from RA patients or from healthy controls that had been diluted 1:4 in PBS with 1 ml of packed gel conjugated to serpin E2 for 16 hours at 4°C on a rocking mixer. The gel was washed 6 times with 2 ml of PBS. For elution of autoantibodies, the gel was incubated with elution buffer (1 ml of 0.1*M* $Na_2CO_3/NaHCO_3$, pH 10.7) for 20 minutes at room temperature. Eluents were neutralized to pH 7 with 0.4*M* HCl, and aliquots were collected for determination of the antibody content by Western blotting. Immediately after elution, the eluents were incubated overnight at 4°C with preblocked Protein A/G Plus–Agarose Beads (Santa Cruz Biotechnology, Heidelberg, Germany). Subsequently, the beads were washed 6 times with distilled water, suspended in distilled water, and used immediately for further procedures.

The presence of isolated antibodies was confirmed by Western blot following every step of antibody purification. To test the reactivity of the isolated anti–serpin E2 autoantibodies, aliquots were used for precipitation of recombinant human serpin E2. Briefly, 25 μl of anti–serpin E2 or irrelevant anti–PTTG-1IP autoantibodies conjugated to Protein A/G Plus–Agarose Beads were incubated overnight with 100 ng of recombinant human serpin E2. Next, the beads were washed 6 times with distilled water, and the amount of precipitated serpin E2 was analyzed by Western blotting. Samples were suspended in Laemmli buffer, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes for immunodetection as previously described (29), using HRP-conjugated goat anti-human IgG antibodies (Jackson ImmunoResearch) or goat anti–serpin E2 (R&D Systems) followed by HRP-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch).

Functional analysis of serpin E2 activity by the urokinase plasminogen activator (uPA) activity assay. The inhibitory activity of recombinant human serpin E2 toward its target, uPA, was determined using a uPA activity assay kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. Briefly, 100 ng of recombinant human serpin E2 was preincubated for 1 hour with Protein A/G Plus–Agarose Beads. The beads were left uncoupled or were coupled to anti–serpin E2 autoantibodies that had been isolated from the sera of healthy subjects (n = 4) and RA patients (n = 4) or to irrelevant anti–PTTG-1IP autoantibodies isolated from the sera of RA patients. To each urokinase reaction, the amount of added antibodies against serpin E2/anti–PTTG-1IP was the same as the amount isolated from 1 ml of sera diluted 1:4. Next, 5 units of uPA, a uPA substrate, and assay buffer were added. In the positive control reaction, only uPA, uPA substrate, and assay buffer were used. Reactions were performed overnight at 37°C. The activity of uPA was then measured with an ELISA reader at 405 nm.

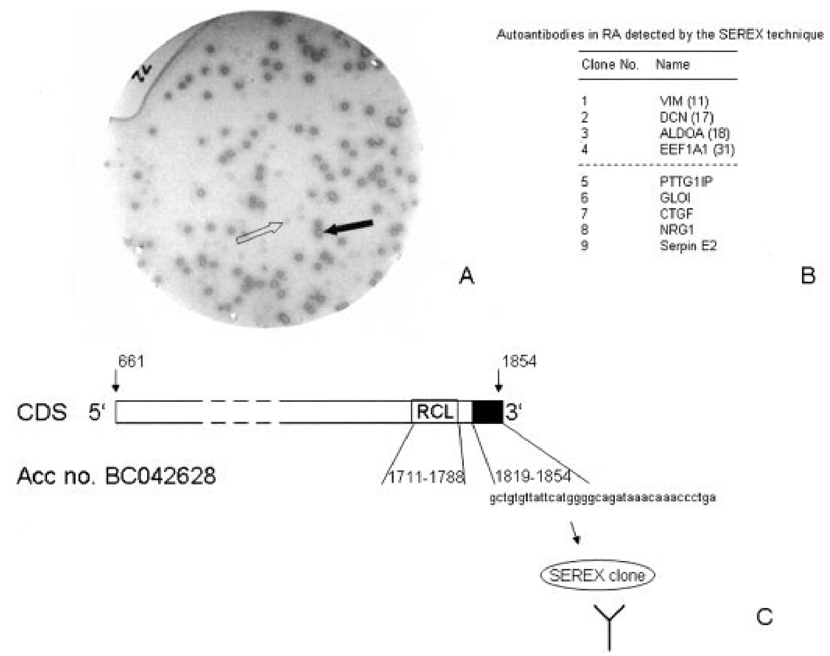


Figure 1. Identification of novel targets for autoantibodies in rheumatoid arthritis (RA) by SEREX technique. A recombinant human cDNA expression library from an RA patient was used for screening by SEREX. RA synovial fluid was used for the identification of novel targets for autoantibodies. **A,** Multiple plaques expressing 1 positive antigen clone that was reactive with synovial fluid from an RA patient as well as 1 negative clone on a nitrocellulose membrane after a second round of screening. Results were scored as positive only when the test clones (**solid arrow**) were clearly distinguishable from the control clones (**open arrow**). **B,** Targets for autoantibodies in RA identified by SEREX. Numbers in parentheses are reference numbers for identified targets previously described as an autoantigen in RA. VIM = vimentin; DCN = decorin; ALDOA = aldolase A; EEF1A1 = eukaryotic translation elongation factor 1 α1; PTTG1IP = pituitary tumor–transforming gene 1–interacting protein; GLOI = glyoxalase I; CTGF = connective tissue growth factor; NRG1 = neuregulin 1 isoform GGF2. **C,** Diagram of a coding sequence (CDS) of serpin E2 (the 661–1,854-bp region of serpin E2 cDNA; accession number BC042628). The autoantibodies from RA synovial fluid were reactive with a peptide encoded by the 1,819–1,854-bp region of serpin E2 cDNA, which is in close proximity to the reactive center loop (RCL) coding region (1,711–1,788 bp).

Statistical analysis. Student's *t*-test and the Mann-Whitney U test were used to compare 2 groups of parametric and nonparametric data, respectively. For assessing correlations, r^2 values were determined with Pearson's correlation coefficient. *P* values less than or equal to 0.05 were considered statistically significant.

RESULTS

Identification of novel autoantibodies in RA synovial fluid. By modifying the SEREX technique and using RA synovial fluid for the identification of novel autoantibodies in RA, we identified 61 immunoreactive clones in the first screening. Positivity of 9 of the clones was confirmed by the second screening (Figures 1A and B). The identified clones included previously described autoreactivities against vimentin, decorin, aldolase A, and the eukaryotic translation elongation factor 1 α1 (EF-1α1). Moreover, we were able to identify novel autoreactive antibodies in RA, namely, anti–PTTG-1IP, anti–glyoxalase I, anti–connective tissue growth factor (anti-CTGF), anti–neuregulin 1 isoform GGF2, and anti–serpin E2.

Serpin E2 as a novel target for autoantibodies in RA. Among other proteins, serpin E2 was recognized by autoantibodies in RA synovial fluid in the SEREX analysis. Serpin E2 is a natural inhibitor of serine proteases, such as plasmin, uPA, thrombin, and trypsin, that show increased activity in RA and are implicated in the pathogenesis of joint destruction. Since serpin E2 has been reported to play a preventive role in cartilage degradation (30), we focused on the role of anti–serpin E2 autoantibodies. The cDNA clone in the plaques reactive with anti–serpin E2 autoantibodies included the 3′ portion of the serpin E2 coding sequence (1,819–1,854 bp). This region of the serpin E2 gene is in close proximity to the region coding for the reactive center loop (RCL), which is essential for the interaction of serpin E2 with serine proteases (Figure 1C).

Expression of serpin E2 in synovial tissues from patients with RA and patients with OA. To investigate the expression levels of serpin E2 in RA and OA synovial tissues, we performed immunohistochemical staining using anti–serpin E2 antibodies (Figures 2A and B). In both RA and OA synovial tissues, serpin E2 was expressed in the cells around blood vessels (median ± SD score 2.0 ± 0.7 in RA versus 1.5 ± 0.7 in OA). Most interestingly, in the synovium of patients with RA (n = 10), serpin E2 was overexpressed in both the lining layer (2.0 ± 0.9 in RA versus 0.5 ± 0.5 in OA; $P \leq 0.01$) and the sublining layer (2.0 ± 0.5 in RA versus 1.0 ± 0.9 in OA; $P \leq 0.01$) (Figure 2C). This pattern differed from that in the synovium of patients with OA, where serpin E2 was present mostly around blood vessels. Thus, serpin E2 was significantly overexpressed in RA synovial tissue.

High levels of anti–serpin E2 autoantibodies in RA synovial fluid. After serpin E2 had been identified as a novel target for autoantibodies using the SEREX technique, the levels of autoantibodies specific for serpin E2 in synovial fluid samples from patients with RA (n = 44), PsA (n = 10), AS (n = 10), and OA (n = 18) were determined by ELISA. Anti–serpin E2 autoantibodies were detected at significantly higher levels in RA synovial fluid (mean ± SD 67 ± 66 AU) than in OA synovial fluid (31 ± 31 AU; $P < 0.05$). Serpin E2–reactive autoantibodies were increased in 28% of the RA synovial fluid samples; in contrast, all of the OA synovial fluid samples were in the normal range (Figure 3A). In both the PsA and the AS patients, anti–serpin E2 autoantibodies were present in 20% of the synovial fluid samples; however, the difference between PsA or AS samples and the

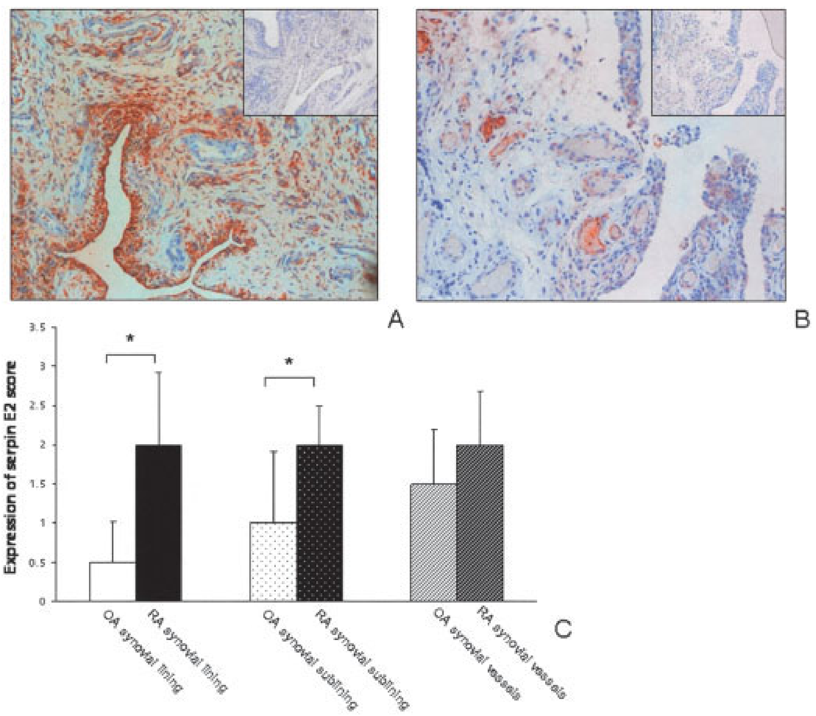
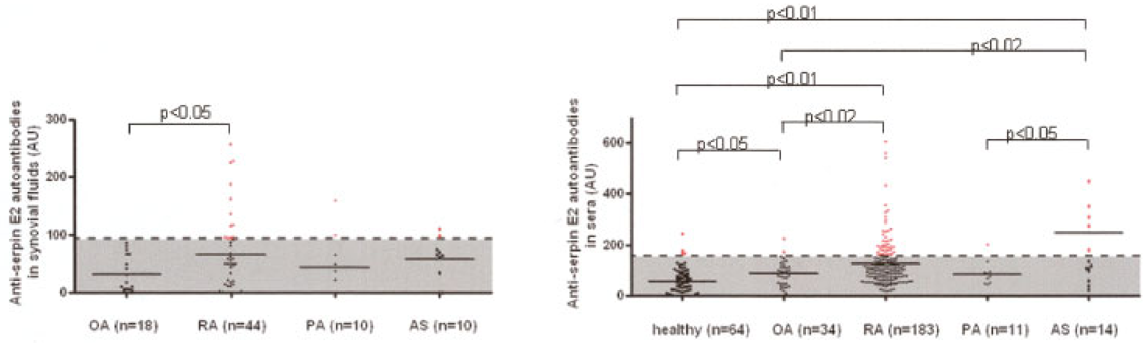


Figure 2. Expression of serpin E2 in synovial tissues from patients with rheumatoid arthritis (RA) and osteoarthritis (OA). **A** and **B,** Immunohistochemical analysis of the expression of serpin E2 in RA and OA synovial tissue sections. Serpin E2 was expressed throughout the synovium in RA tissues (**A**) and mainly around vessels in OA tissues (**B**). **Insets** show the respective IgG controls. (Hematoxylin counterstained; original magnification × 100). **C,** Scoring of immunohistochemically stained sections for the expression of serpin E2 in 10 OA and 10 RA synovial tissue sections. The lining layer (left pair of bars), sublining layer (middle pair of bars), and vessels (right pair of bars) were scored individually. Values are the median and SD. * = $P \leq 0.05$ by Mann-Whitney U test.



Synovial fluids	OA	RA	PA	AS
Mean anti-serpin E2 (AU)	31	67	43	58
% positive	0	28	20	20

A

Sera	healthy	OA	RA	PA	AS
Mean anti-serpin E2 (AU)	61	86	125	81	247
% positive	6	6	22	9	33

B

Figure 3. Levels of anti–serpin E2 autoantibodies in **A,** synovial fluid and **B,** serum from patients with osteoarthritis (OA), rheumatoid arthritis (RA), psoriatic arthritis (PA), and ankylosing spondylitis (AS), as well as in sera from healthy controls. Levels of autoantibodies reactive with recombinant human serpin E2 were determined by enzyme-linked immunosorbent assay. Each data point represents a single subject; horizontal lines show the mean. Broken horizontal line represents the cutoff value for positivity; red symbols represent subjects positive for anti–serpin E2. *P* values were calculated using Student's *t*-test (significant at $P \leq 0.05$). Tables at the bottom summarize the data. AU = arbitrary units.

control OA synovial fluid samples did not reach statistical significance (mean ± SD 43 ± 53 AU in PsA and 58 ± 37 AU in AS). The RA patients, therefore, had a higher prevalence of anti–serpin E2 autoantibodies in synovial fluid than did the OA patients.

High levels of anti–serpin E2 antibodies in RA patient sera. Next, we analyzed whether anti–serpin E2 autoantibodies were also present in sera. Serum samples derived from the peripheral blood of RA patients (n = 183), OA patients (n = 34), PsA patients (n = 11), AS patients (n = 14), and healthy individuals (n = 64) were tested for the presence of anti–serpin E2 autoantibodies. Anti–serpin E2 autoantibodies were detected at significantly higher levels in RA patient sera (mean ± SD 125 ± 90 AU) than in OA patient sera (86 ± 51 AU; $P < 0.02$) and normal sera (61 ± 51 AU; $P < 0.01$). Anti–serpin E2 autoantibodies were detected in 22% of serum samples from patients with RA. This differed significantly from that in OA patients and healthy individuals; both groups showed 6% positive sera (Figure 3B). Furthermore, in RF-negative RA patients, 16% of the serum samples were positive for anti–serpin E2 autoantibodies. The levels of anti–serpin E2 autoantibodies in the anti-CCP–positive and anti-CCP–negative subgroups were not statistically different. Of the PsA sera, 9% were positive for anti–serpin E2 autoantibodies (mean ± SD 81 ± 53 AU); this value did not differ significantly from that in the control OA sera. In contrast, anti–serpin E2 autoantibodies were present in 33% of AS sera (247 ± 304 AU), and the values in this group differed significantly from those in the healthy subjects ($P < 0.01$), the OA patients ($P < 0.02$), and the PsA patients ($P < 0.05$).

Although autoantibodies against serpin E2 were detected by the screening procedure using synovial fluid samples, they were also similarly detected using serum samples. Thus, RA patients showed significantly higher levels of anti–serpin E2 autoantibodies than did OA patients and healthy controls.

Assessment of the functional role of anti–serpin E2 autoantibodies. Since serpin E2 has profound effects on the remodeling of the extracellular matrix and since the autoantibodies bind to a region of serpin E2 that lies in proximity to the RCL, we analyzed the function of the autoantibodies on the activity of serpin E2. To evaluate whether the inhibitory function of serpin E2 is influenced by the presence of specific anti–serpin E2 autoantibodies, we isolated anti–serpin E2 antibodies from

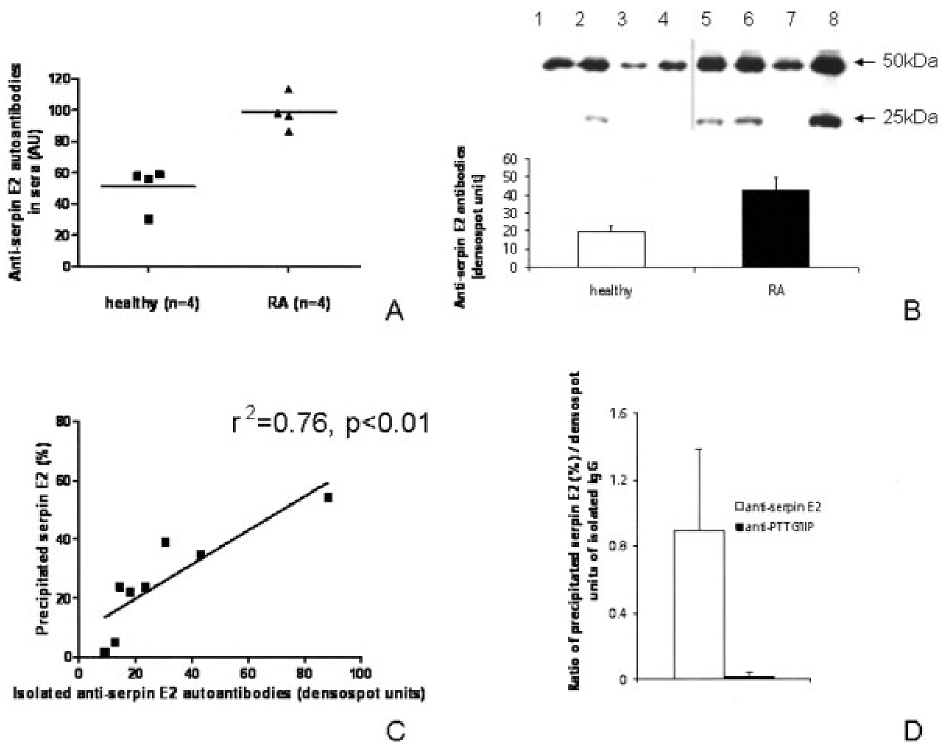


Figure 4. Precipitation of serpin E2 by anti–serpin E2 autoantibodies isolated from sera. **A,** Levels of autoantibodies reactive with recombinant human serpin E2 in serum samples from healthy subjects and from patients with rheumatoid arthritis (RA) whose samples were used to isolate the anti–serpin E2 autoantibodies. Each data point represents a single subject; horizontal lines show the mean. AU = arbitrary units. **B,** Detection of autoantibodies by Western blotting with anti-human IgG antibodies (top). Shown are anti–serpin E2 autoantibodies isolated from the sera of healthy subjects (lanes 1–4) or patients with RA (lanes 5–8) and anti–pituitary tumor–transforming gene 1–interacting protein (anti–PTTG-1IP) antibodies isolated from an RA patient serum (lane 9). Arrows indicate IgG heavy and light chains of expected sizes. Anti–serpin E2 autoantibody levels in the respective groups of blots were quantified by densitometry (bottom). Values are the mean and SD. **C,** Correlation between the amount of anti–serpin E2 autoantibodies isolated and the percentage of serpin E2 precipitated. **D,** Ratio of the percentage of precipitated serpin E2 per densospot unit of isolated anti–serpin E2 autoantibodies and per densospot unit of isolated anti–PTTG-1IP autoantibodies. Values are the mean and SD.

the sera of patients with RA. As a control, we used sera from healthy individuals. Serpin E2 was immobilized on Sepharose beads and incubated with diluted sera from RA patients or healthy controls. The antibodies were eluted and then purified from the eluents by binding to Protein A/G Plus–Agarose Beads. The isolated antibodies were quantified by Western blotting.

As expected, more anti–serpin E2 autoantibodies were isolated from the sera of RA patients (n = 4; mean ± SD 42 ± 7 densospot units) as compared with the sera of healthy individuals (n = 4; 19 ± 3 densospot units) (Figures 4A and B). The isolated anti–serpin E2 autoantibodies precipitated serpin E2 according to their concentration ($r^2 = 0.76$, $P < 0.01$) (Figure 4C). We also tested whether control irrelevant (anti–PTTG-1IP) autoantibodies isolated from RA sera (n = 2) could precipitate serpin E2. As expected, the irrelevant anti–PTTG-1IP autoantibodies did not precipitate serpin E2 (Figure 4D).

Since uPA is a serine protease that is inhibited by serpin E2, we investigated the ability of serpin E2 to inhibit the activity of uPA in the presence of anti–serpin E2 autoantibodies isolated from the sera of patients with RA. We analyzed the activity of uPA in the presence of recombinant human serpin E2 that had or had not been preincubated with anti–serpin E2–specific autoantibodies isolated from RA patients. As expected, recombinant human serpin E2 (500 ng/ml) inhibited the activity of uPA (Figure 5A). Most importantly, in the presence of serpin E2–specific autoantibodies isolated from RA patient sera, the inhibition of uPA activity by serpin E2 was diminished by a mean ± SD of 69 ± 8% (n = 4). Anti–serpin E2 antibodies isolated from normal sera diminished the inhibition of the uPA activity by serpin

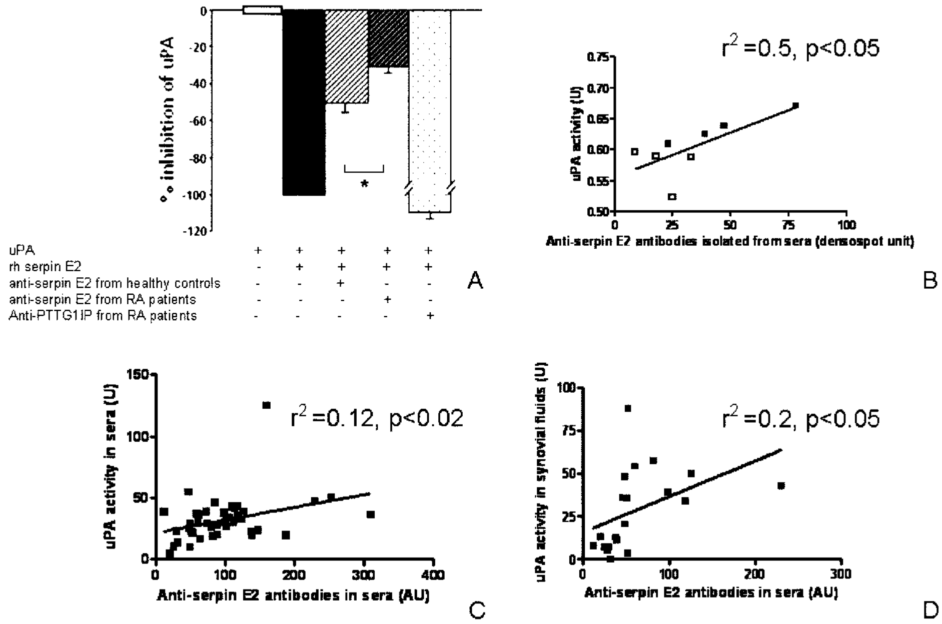


Figure 5. Correlation between anti–serpin E2 autoantibody levels and urokinase plasminogen activator (uPA) activity. **A,** Activity of uPA, as measured in a uPA activity assay and expressed as the percentage inhibition of uPA. Down-regulation of uPA activity in the absence of recombinant human (rh) serpin E2 (open bar), in the presence of recombinant human serpin E2 (set at 100%) (solid bar), in the presence of recombinant human serpin E2 preincubated with anti–serpin E2 antibodies isolated from the sera of normal subjects (n = 4) (open striped bar), or from the sera of rheumatoid arthritis (RA) patients (n = 4) (shaded striped bar), and in the presence of recombinant human serpin E2 preincubated with anti–pituitary tumor–transforming gene 1–interacting protein (anti–PTTG-1IP) antibodies isolated from the sera of RA patients (n = 2) (open dotted bar) are shown. Values are the mean and SD. * = $P < 0.05$ by Mann-Whitney U test. **B,** Correlation between the levels of anti–serpin E2 autoantibodies isolated from the sera of RA patients (solid squares) and normal controls (open squares) (n = 4 per group) and the activity of uPA in the presence of recombinant human serpin E2. **C,** Correlation between the levels of anti–serpin E2 antibodies and the activity of uPA measured in sera from RA patients (n = 38), PsA patients (n = 5), and AS patients (n = 5). AU = arbitrary units. **D,** Correlation between the levels of anti–serpin E2 antibodies measured in serum samples and the activity of uPA in synovial fluid samples from the same patients: 10 with RA, 5 with PsA, and 5 with AS.

E2 by 49 ± 10% (n = 4). Thus, the reduction in the inhibitory effect of recombinant human serpin E2 in the presence of autoantibodies isolated from RA patients was significantly higher than that in the presence of autoantibodies isolated from healthy individuals ($P <$ 0.05) (Figure 5A).

In the presence of irrelevant (anti–PTTG-1IP) autoantibodies isolated from RA sera (n = 2), the inhibitory activity of serpin E2 toward uPA was not decreased. Since isolated anti–serpin E2 autoantibodies from different sera impaired the activity of serpin E2 to different extents, we correlated the amount of isolated antibodies and the respective values for the uPA activity. Increasing quantities of antibodies, as determined by densitometry, correlated significantly with uPA activity ($r^2 = 0.5$, $P < 0.05$) (Figure 5B). From these experiments, we conclude that anti–serpin E2 autoantibodies impair the inhibitory function of recombinant human serpin E2 in vitro in a dose-dependent manner.

Correlation of serum anti–serpin E2 autoantibody levels with uPA activity in serum and synovial fluid in vivo. Based on the results showing in vitro the function of isolated anti–serpin E2 autoantibodies on the inhibitory activity of recombinant human serpin E2, we next investigated the function of these autoantibodies in vivo. In these analyses, we examined the uPA activity and the anti–serpin E2 concentration in serum samples from 38 RA patients, 5 PsA patients, and 5 AS patients and synovial fluid samples from 10 RA patients, 5 PsA patients, and 5 AS patients. We found that, indeed, high levels of anti–serpin E2 autoantibodies corresponded to high levels of uPA activity in sera ($r^2 = 0.12$, $P < 0.02$), indicating that in vivo, serpin E2 is a less active inhibitor in the presence of anti–serpin E2 autoantibodies (Figure 5C). In contrast, the levels of anti–serpin E2 autoantibodies in synovial fluid did not correlate significantly with the activity of uPA in synovial fluid (data not shown). In addition, the quantities of anti–serpin E2 autoantibodies found in sera correlated significantly with the activity of uPA in synovial fluids ($r^2 = 0.2$, $P < 0.05$) (Figure 5D). In conclusion, the levels of anti–serpin E2 autoantibodies in serum were associated with the levels of activity of uPA in serum and synovial fluid.

DISCUSSION

Using the SEREX screening technique, we identified several novel targets for autoantibodies in RA. Among the clones we identified were decorin, vimentin, and aldolase A, for which specific autoantibodies have already been described in patients with RA (11,17,18). Another antigen, EF-1α1, has previously been shown to be immunoreactive in Felty's syndrome and has also been related to RA (31). Moreover, we were able to identify previously unknown autoreactive antibodies in RA synovial fluid samples, namely, autoantibodies directed toward PTTG-1IP, glyoxalase I, CTGF, neuregulin 1 isoform GGF2, and serpin E2.

Serine proteases, such as plasmin, tissue plasminogen activator (tPA), uPA, thrombin, and trypsin, show increased activity in RA patients and are thought to contribute to the pathogenesis of the disease (32–36). In a collagen-induced mouse model of RA, plasmin was shown to be an essential component of the early phase of pathogenesis (37). Thrombin is known to induce angiogenesis, fibrin formation, and inflammation (38,39), which are also the primary events in the joint destruction in RA. RA synovial tissues were shown to exhibit considerably increased uPA activity in the lining layer, and RA synoviocytes were shown to be more prone to uPA-challenged invasion and proliferation (36,40).

Specific inhibitors of serine proteases, such as antithrombin, antiplasmin, and plasminogen activator inhibitor 1 (PAI-1), belong to the family of serine protease inhibitors (serpins). Serpins are classified into 16 groups according to phylogenetic analysis, with members present in higher animals, nematodes, insects, plants, and viruses. The majority of the serpin family members are active serine protease inhibitors with the reactive center loop–active site located in the C-terminal portion. The RCL forms a bait that is targeted by a serine protease. After the protease cleaves the RCL domain, it is bound covalently on the catalytic serine residue and is irreversibly inhibited. Next, the protease bound to serpin is internalized and degraded.

Serpin E2, which is also called protease nexin 1/glial-derived nexin, is classified to clade E of the serpin family, together with PAI-1 and myxoma virus serpin Serp-1. Serpin E2 regulates matrix accumulation and coagulation by inhibiting thrombin, plasmin, tPA, and uPA. Serpin E2 was shown to be induced by proinflammatory cytokines in different cell types. Interleukin-1β (IL-1β), transforming growth factor β, and tumor necrosis factor α (TNFα)–induced serpin E2 in neurons and muscle cells (41,42), chronic exposure to TNFα in rat fibroblast-like synoviocytes and mouse endothelial cells (43,44), and stimulation of human monocytes with lipopolysaccharide were shown to up-regulate the expression of serpin E2 (45). Recently, serpin E2 was detected at high levels in atherosclerotic plaques and was suggested to play a protective role against aggression of proteases under inflammatory conditions (45). Most interestingly, serpin E2 indeed prevented IL-1β/basic fibroblast growth factor–induced articular cartilage loss through the inhibition of plasmin, thus preventing the subsequent activation of matrix metalloproteinases (MMPs) in rabbits (30).

Given the protective role of serpin E2 against cartilage loss by inhibiting plasmin and by averting the subsequent activation of MMPs (30,46), we focused on analyzing autoantibodies against serpin E2 in RA.

We have established an ELISA with which we measured the levels of anti–serpin E2 autoantibodies in serum and synovial fluid from patients with RA, patients with OA, and healthy individuals. In the present study, we showed that patients with RA have high levels of anti–serpin E2 autoantibodies. It might be argued that RF present in RA patient sera could react with the anti–serpin E2 autoantibodies and thereby amplify the signal obtained in ELISA. We therefore analyzed RF-negative sera from RA patients ($n = 37$). The levels of anti–serpin E2 autoantibodies detected in RF-negative sera from RA patients were also significantly higher than those in healthy controls (data not shown).

To investigate the functional significance of elevated levels of anti–serpin E2 in patients with RA, we tested the inhibitory activity of recombinant human serpin E2 in the presence of anti–serpin E2 autoantibodies isolated from human serum. We showed that the activity of serpin E2 in vitro was decreased by anti–serpin E2 autoantibodies isolated from sera of patients with RA. Fewer anti–serpin E2 autoantibodies were isolated from the sera of healthy individuals, which was consistent with the results from the ELISA. Anti–serpin E2 autoantibodies isolated from the sera of healthy individuals also interfered with the activity of serpin E2, but to a lesser extent, according to their lower concentration. We conclude that the pathologically elevated levels of anti–serpin E2 autoantibodies found in RA patients could lead to the increased activity of serine proteases in this disease.

Serum levels of anti–serpin E2 autoantibodies correlated significantly with the activity of uPA in serum and in synovial fluid. Higher levels of anti–serpin E2 autoantibodies were detected in serum than in synovial fluid from RA patients. This could be due to infiltration and attachment of the anti–serpin E2 autoantibodies to serpin E2, which is overexpressed in RA synovial tissue; this would result in a lowering of the anti–serpin E2 levels in synovial fluid. This possibility is supported by the results showing that the serum levels of anti–serpin E2 autoantibodies correlated significantly with uPA activity in synovial fluid, whereas we did not observe a correlation between uPA activity and the levels of synovial fluid anti–serpin E2.

It remains to be elucidated whether autoantibodies reduce the activity of serpin E2 through binding and blocking of the RCL domain, which is essential for activity, through changing the conformation of serpin E2 or through another mechanism. Analysis of the sequence of the cDNA coding for a fragment of serpin E2 that is reactive with the sera of RA patients by the SEREX method showed that the autoantibodies bind to 10 amino acids in the C-terminal domain of serpin E2. Since the RCL responsible for the inhibition of serine proteases is located in the C-terminus, it is possible that the autoantibodies that bind to the C-terminal portion of serpin E2 could block the interaction of serpin E2 with the proteases.

In RA synovial tissues, serpin E2 was expressed in cells around blood vessels, in the lining layer, and in the sublining layer of the synovium. In contrast, in OA control synovial tissues, serpin E2 was mainly expressed by cells around blood vessels. The expression of serpin E2 in vessels has been described in normal human arteries, but not in plasma or in circulating blood, unlike other serpins, such as antithrombin III, antitrypsin, and antichymotrypsin, which function as serine protease inhibitors in the regulation of coagulation cascades (45). Serpin E2 localized in blood vessels could be responsible for the regulation of thrombosis or coagulation at the tissue level or the cellular level. The increased expression and the special localization of serpin E2 in the synovium of patients with RA could indicate that a counterregulatory mechanism is activated to decrease the activity of proteases and that this putative defense mechanism is at least partly inhibited by anti–serpin E2 autoantibodies.

It is not known whether the direct blocking effect of anti–serpin E2 autoantibodies on the function of serpin E2 is an isolated feature of this specific antigen–antibody complex. It is possible that other antigens are also modified in their activities or properties when bound to specific autoantibodies. From this viewpoint, the production of autoantibodies could play an important role in the pathogenesis of RA, rather than being an epiphenomenon. Pullerits et al (47) recently reported that the presence of autoantibodies specific for receptor for advanced glycation end products (RAGE) coincides with the amelioration of erosions in RA, which suggests a protective role of anti-RAGE autoantibodies against inflammation in the synovium. Here, in contrast, we found that autoantibodies specific for serpin E2 might worsen the disease, since autoantibodies isolated from the sera of patients with RA reversed the protective inhibitory activity of serpin E2. Anti–serpin E2 autoantibodies might therefore favor the proteolytic milieu in RA joints, as well as enhance inflammation, since it has been reported that increased serine protease activity intensifies inflammation (48). It would therefore be interesting to investigate whether RA patients with increased levels of anti–serpin E2 autoantibodies respond to B cell depletion therapy with, for example, anti-CD20 antibodies, as has been shown with some autoantibodies in systemic lupus erythematosus patients.

It must also be considered that serpin E2 inhibits a wide range of proteases involved in the pathogenesis of RA. Thus, it is probable that the presence of autoantibodies specific for serpin E2 could have a greater effect on the destruction of cartilage than just increasing the activity of uPA (Figure 6). However, it is difficult to evaluate the extent to which anti–serpin E2 antibodies

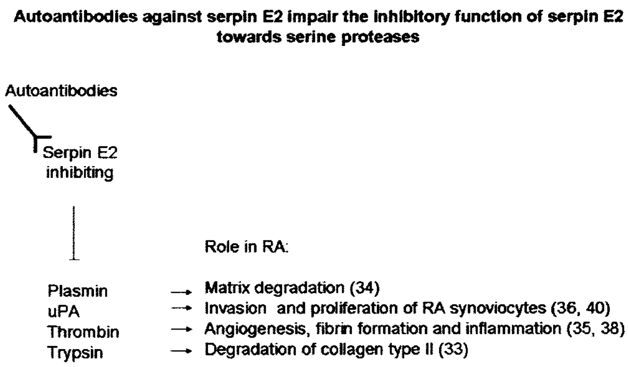


Figure 6. Hypothetical pathways influenced by the interaction of serpin E2 with anti–serpin E2 autoantibodies. The occurrence of anti–serpin E2 autoantibodies that have the ability to interfere with the inhibitory activity of serpin E2 might cause a different outcome in rheumatoid arthritis (RA). Numbers in parentheses are reference numbers. uPA = urokinase plasminogen activator.

influence cartilage destruction through the in vivo impairment of the function of serpin E2. In the present study at least, we showed that autoantibodies isolated from the sera of RA patients impaired the inhibition of uPA activity by serpin E2 in vitro and that high anti–serpin E2 autoantibody levels correlated with high uPA activity in vivo. Moreover, since serpin E2 is abundantly present in atherosclerotic plaques (45) and since vascular changes are common in RA patients (49), we conclude that the anti–serpin E2 autoantibodies could also play a role in the formation of the atherosclerotic plaques. We are currently performing studies to address this possibility. To our knowledge, this study is the first to show a distinct effect of an autoantibody that might be related to the pathogenesis of RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Jüngel had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Maciejewska-Rodrigues, Al-Shamisi, Ospelt, Jäger, R. E. Gay, Knuth, Neidhart, Jüngel.

Acquisition of data. Maciejewska-Rodrigues, Bouton, Plant, Distler.

Analysis and interpretation of data. Maciejewska-Rodrigues, Hemmatazad, Cope, Charles, Distler, Michel, S. Gay, Jüngel.

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3 Epigenetics and rheumatoid arthritis: The role of SENP1 in the regulation of MMP-1 expression

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Epigenetics and rheumatoid arthritis: The role of SENP1 in the regulation of MMP-1 expression☆

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ABSTRACT

The aggressive phenotype of RA synovial fibroblasts (RASF) is characterised by the increased expression of matrix metalloproteinase (MMP)-1 as well as the small ubiquitin like modifier (SUMO)-1 and decreased expression of SUMO-specific protease SENP1. Since we showed an increased activity of acetyltransferases in this autoimmune disease, we wanted to analyze whether this affects the expression of MMP-1 and can be reversed by the reconstitution of SENP1.

In RASF, the acetylation of histone H4 was significantly increased in the distal region of the MMP-1 promoter by 274 ± 36% compared to OASF. Most interestingly, overexpression of SENP1 in RASF decreased acetylation specifically in this region by 51 ± 0.5% and globally by 73 ± 11%. Furthermore, the overexpression of SENP1 resulted in a downregulation of MMP-1 at both the mRNA (58 ± 7%) and protein levels (28 ± 6%), significantly reduced the invasiveness of RASF (from 34 ± 9% to 2 ± 2%) and led to an accumulation of histone deacetylase 4 (HDAC4) on the MMP-1 promoter (197 ± 36%). Interestingly, SENP1 failed to modulate the expression of MMP-1 in the cells silenced for HDAC4. This is the first study linking the SUMOylation pathway and the production of MMP-1 to an epigenetic control mechanism mediated through histone acetylation which has a functional consequence for the invasiveness of RASF.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by persistent inflammatory processes in the joints resulting in a progressive articular destruction associated with the loss of joint function and disability [1,2]. RA synovial fibroblasts (RASF) are intrinsically activated and therefore invade into cartilage by excessive expression of matrix degrading enzymes such as matrix metalloproteinases (MMPs) MMP-1, 3, 9, 10 and 13 [3–7]. MMP-1 is one of the most important MMPs since it is responsible for cartilage destruction by cleaving collagen type II.

Recently, our group could show that in RA synovial tissues the balance of activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs) is strongly shifted towards histone acetylation [8], which is associated with increased transcription rates [9,10]. This posttranslational modification of histones belongs to epigenetic modifications regulating gene expression and offers us new mechanism for therapeutic strategies. Another posttranslational modification described in RA is SUMOylation [11,12]. We could show that RASF have intrinsically high levels of small ubiquitin like modifier (SUMO)−1 paralleled by decreased levels of its specific protease (SENP1) which renders them apoptosis-resistant.

Whereas posttranslational modifications such as acetylation/deacetylation directly influence gene transcription, SUMO-1 can

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influence the properties of its substrates such as stability, localization, interaction with other proteins and activity [13–15]. Histone deacetylase 4 (HDAC4) is known to be modified by SUMO-1 and this is thought to modulate its activity as a negative regulator of transcription [16]. There is, however, uncertainty regarding the influence of SUMOylation on the activity of HDAC4. Kirsh et al. reported that a SUMOylation-deficient mutant of HDAC4 shows only a slightly impaired ability to repress the transcription while the nuclear transport of HDAC4 results from the modification by SUMO-1 [16]. This suggests that sumoylation of HDAC4 could rather contribute to the change in the localization of HDAC4 than influence the activity directly.

Since SUMO-1 is a modifier of a wide range of transcription and epigenetic factors, we studied here to which extent the activated phenotype of RASF characterised by excessive production of MMP-1 can be modified by epigenetic changes driven by desumoylation. This is the first study linking SUMOylation and the production of MMP-1 to an epigenetic control mechanism mediated through histone acetylation, and therefore, it supports the idea that epigenetic modulations are involved in RA [17].

2. Materials and methods

2.1. Cells and patients

Synovial fibroblasts were obtained from RA ($n = 8$) and OA patients ($n = 3$) undergoing joint replacement surgery and grown in DMEM with 10% FCS until passages 4–6 as described [18]. All RA patients fulfilled the American College of Rheumatology criteria for RA [19].

2.2. Expression vectors, siRNA, and transfection

A GFP-tagged SENP1 expression vector (SENP1-pEGF-C1) was generated by subcloning SENP1 into pEGF-C1 as described previously [12]. HDAC4 siGENOME SMARTpool was purchased from Dharmacon, Lausanne, Switzerland and siRNA HDAC4 from Santa Cruz, Heidelberg, Germany. The mock vector pEGF-C1 or mismatch oligonucleotides (Qiagen, Hombrechtikon, Switzerland) were used as controls. Transfection of the cells with SENP1 and HDAC4 siRNA were performed by AMAXA nucleofection as described previously [20].

2.3. Chromatin immunoprecipitation

In a typical experiment, 0.5 million of SF were harvested, chromatin was crosslinked with 1% formaldehyde for 10 min and the reaction was quenched by adding glycine. Cells were washed twice, snap frozen and stored at -80 °C or immediately used. Chromatin sonication was performed on ice in SDS lysis buffer containing protease inhibitors (1× protease inhibitors cocktail, Roche, Basel, Switzerland), 5 mM sodium butyrate (Sigma, Buchs SG, Switzerland) and 2.5 μM trichostatin A (TSA, Sigma) using Bandelin sonopuls UW 2070 sonicator and a program of 4 cycles of 10 s at 57% power. The efficiency of the sonication of the chromatin was analysed by gel electophoresis. 10% of the sample were kept as input. Chromatin was diluted in ChIP dilution buffer and pre-cleaned by incubation with normal rabbit serum and pre-blocked Protein A agarose beads (Upstate/Millipore, Zug, Switzerland) for at least 2 h. Chromatin immunoprecipitation was performed by overnight incubation with anti-acetyl H4 antibodies, rabbit anti-HDAC4 antibodies (Santa Cruz) or isotype IgG as a negative control and followed by at least 4 h incubation with Protein A agarose beads. Subsequently, immunoprecipitates were washed with buffers containing protease inhibitors and sodium butyrate and incubated for 20 min with the elution buffer containing proteinase K. Reverse crosslinking was performed overnight at 64 °C and the DNA was purified using the QIAquick gel extraction kit (Qiagen).

2.4. RNA extraction

Total RNA was isolated and converted into cDNA and gene expression was quantified by Real-time PCR as described [20,21].

2.5. Real-time polymerase chain reaction

2.5.1. Quantification of mRNA

Expression of MMP-1 was assessed using self designed previously published primers and probe [22] and HDAC4 mRNA was quantified by Real-time PCR using SybrGreen primers: fwd 5′- TGT ACG ACG CCA AAG ATG AC -3′, rev 5′- CGG TTC AGA AGC TGT TTT CC -3′. To confirm specific amplification by SybrGreen PCR, dissociation curve analysis was performed and water controls were included for the primer pair and NRT controls were analysed for all samples. The amounts of loaded cDNA were normalized using a predeveloped 18S assay (PE Applied Biosystems). Differential gene expression was calculated with the threshold cycle (Ct) and the comparative Ct method for relative quantification. All reactions were performed in duplicates.

2.5.2. Quantification of ChIP

Immunoprecipitated promoter fragments were quantified by Real-time PCR using SybrGreen primers. The primer sequences used are as follows: for **MMP-1 ChIP1** fwd 5′- TGG GAT ATT GGA GCA GCA AG -3′, rev 5′- AGC TGT GCA TAC TGG CCT TT -3′; for **MMP-1 ChIP2** fwd 5′- TAA GGG AAG CCA TGG TGC TA -3′, rev 5′- AGG TTC CCT TCT GCC TTT CT -3′; for **MMP-1 ChIP3** fwd 5′- TGA CTG GGA AGT GGA AAC CT -3′, rev 5′- GCC TGC AAT GGT GAG TCAT -3. To confirm specific amplification by SybrGreen PCR, dissociation curve analysis was performed and water controls were included for each primer pair. The amounts of immunoprecipitated fragments of genomic DNA were normalized to the input DNA. Differential immunoprecipitation of promoter fragments was calculated with the threshold cycle (Ct) and the comparative Ct method for relative quantification. All reactions were performed in duplicates.

2.6. Immunofluorescence

Immediately after transfection, cells were plated on chamber slides and incubated for 48 h. Cells were washed with PBS. Fixation was performed using 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were washed with PBS and permeabilized with freshly prepared 0.1% triton X-100 (Beucher & Hobein, AG, Zurich, Switzerland) in PBS for 10 min at room temperature. All the solutions used in the following steps contained 0.1% triton X-100. Cells were blocked for 1 h using 8% BSA in PBS and incubated overnight with rabbit anti-acetyl H4 antibodies (Upstate/Millipore) or an IgG isotype in 3% BSA in PBS. Cells were washed and incubated with Cy™3 conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch). After washing cells were mounted. The detection of SENP1-pEGF-C1 and the acetylated histone H4 was performed by fluorescence microscopy.

2.7. Flow cytometry analysis (FACS)

Cells were permeabilised with 0.4% triton X-100 and incubated with rabbit anti-acetyl H4 (Upstate/Millipore) antibodies and followed by incubation with R-Phycoerythrin-labeled donkey anti-rabbit antibodies (Jackson ImmunoResearch). Unbound antibodies were removed by two washing steps. In control experiments primary antibodies were replaced by a mouse IgG isotype in the same concentration. Measurements were performed using 10.000 cells per sample at "low-flow" modus using FACSCalibur (BD Biosciences, Allschwil Switzerland).

2.8. Acid extraction of histones

Following transfection, cells splited into two portions and the first portion was subjected to acid extraction of histones, while second portion was lysed in Lemmli buffer and used to assess the efficiency of the transfection. The acid extraction was performed as previously described [23]. Briefly, nuclear extracts were prepared by cell lysis in cold hypotonic lysis buffer (10 mM Tris–Cl pH 8.0, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, containing 1× protease inhibitors cocktail (Roche), 1.25 mg/ml sodium butyrate (Sigma) and 2 μM TSA (Sigma)). The nuclei were resuspended in 0.4 N H_2SO_4 and incubated on a rotator at 4 °C for 1 h. Next, histones were precipitated overnight by 33% trichloroacetic acid. The histone pellet was washed with ice-cold acetone and resuspended in distilled water.

2.9. Western blot

Following transfection, lysates were suspended in 2× concentrated Lemmli buffer (100 m*M* Tris HCl [pH 6.8], 40% glycerol, 10% sodium dodecyl sulfate [SDS], 0.7 M β-mercaptoethanol, and 0.0005% bromphenol blue) and separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes as previously described [18]. Membranes were blocked with 5% milk for 1 h at RT and then incubated overnight with rabbit anti-acetylated histone H4 (Upstate/Millipore), rabbit anti-SENP1 (Chemicon, Asperg, Germany) or mouse anti-MMP-1 (R&D Systems). As secondary reagents, HRP-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch, Magden, Switzerland) or HRP-conjugated rabbit anti-mouse IgG antibodies (DAKO, Baar, Switzerland) were used, and signals were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Otelfingen, Switzerland). For normalization, membranes were stripped and probed with mouse anti-human α-tubulin (Sigma), mouse anti-paxillin antibodies (Neomarkers, Basel, Switzerland) or rabbit anti-histone H4 (Upstate/Millipore). Evaluation of the expression of specific proteins was performed using the Alpha imager Software system (Alpha Innotech, San Leandro, CA) via pixel quantification of the electronic image.

2.10. Cell invasion assay

Investigation of synovial fibroblast invasion in vitro was performed using the recently established matrix associated trans-epithelial resistance invasion (MATRIN) assay as described [24]. This is a highly sensitive electrophysiological technique that is based on the measurement of the electrical resistance of a monolayer of the C7 subclone of Madin–Darby canine kidney cells (MDCK-C7). MDCK-C7 cells were grown in MEM on the back of a 0.4-μm filter cup. Pooled RASF from two patients were transfected with SENP1 (SENP1-pEGF-C1) or the empty vector (pEGFP-C1), and 5×10^5 cells were seeded onto a collagen matrix consisting of 97% type I collagen and 3% type III collagen that was used to coat the top of the filter cup. The resistance across this monolayer was measured with a STX2 electrode. The invasion was recorded 12, 19 and 23 h after seeding the cells. Measurement for all cells was performed in triplicates.

2.11. Statistical analysis

All data are expressed as mean ± SEM. Mann–Whitney *U*-test was used for comparison between two groups of data. *p* values lower or equal to 5% were considered statistically significant ($p \leq 0.05$).

3. Results

3.1. RASF have higher intrinsic levels of acetylated histone H4 in the promoter of MMP-1 than OASF

Since we could show that the acetylation of histones is increased in RA [8] we investigated whether there is hyperacetylation of histones in the promoter of MMP-1 in RASF compared to OASF contributing to its overexpression in RA. We performed chromatin immunoprecipitation in RASF and OASF using anti-acetyl H4 antibodies and analysed 3 different regions spread along the promoter of MMP-1. Most interestingly, we found that in the upstream region −1524 to −1464 from the transcription starting point of the MMP-1 promoter, the levels of acetylation of histone H4 were significantly increased in RASF when compared with OASF (percent change 274 ± SEM 36%, $n = 3$, $p \leq 0.05$, Fig. 1A). The levels of acetylated histone H4 in the −552 to −488 upstream region from the transcription starting point were slightly lower in RA when compared to OA (22 ± 9%, $n = 3$, $p \leq 0.05$). There was no difference in acetylation of histones in the region −179 to −130 upstream from the transcription starting point. Therefore, we hypothesised that hyperacetylation of the MMP-1 promoter could result in an intrinsically upregulated production of MMP-1 in RASF.

3.2. SENP1 overexpression leads to a decrease in acetylation of histone H4 in the promoter of MMP-1

RASF are characterised by increased SUMOylation due to an upregulation of SUMO-1 and the downregulation of SENP1. To reverse this phenotype we overexpressed SENP1 in RASF using an expression construct of SENP1 (SENP1-pEGF-C1) or the empty vector (mock transfected, pEGF-C1) and analysed the acetylation status of the MMP-1 promoter. We found that the levels of acetylated histone H4 in the region at −1524 to −1464 bp from the transcription starting point were significantly downregulated by the overexpression of SENP1 by 51 ± 0.5% ($n = 3$, $p \leq 0.05$, Fig. 1B). The levels of acetylated histone H4 in the two other analysed regions were not significantly different in cells overexpressing SENP1.

3.3. Desumoylation decreases the global levels of acetylated histone H4

Since SENP1 overexpression decreased the acetylation level in the promoter of MMP-1 we were interested whether the decrease in acetylation of histones is also a global phenomenon. Thus, we compared the global levels of acetylated histone H4 in the cells transfected with SENP1-pEGF-C1 or the mock vector. As visualized by immunofluorescence, RASF transfected with SENP1-pEGF-C1 revealed lower global levels of acetylated histone H4 when compared both to nontransfected or GFP transfected cells ($n = 3$, Fig. 2A). Using flow cytometry analysis we could further confirm that the global levels of H4-acetylation were significantly decreased by 73 ± SEM 11% when compared to pEGF-C1 cells ($n = 3$, $p < 0.05$, Fig. 2B). In addition, using Western blot we could confirm that the 3-fold overexpression of SENP1 (Fig. 2C) leads to a decrease in acetylation of histone H4 (by 51 ± SEM 4%, Fig. 2D).

3.4. Overexpression of SENP1 results in a downregulation of MMP-1 in RASF

After we observed decreased acetylation of histones in the MMP-1 promoter in RASF overexpressing SENP1, we wanted to

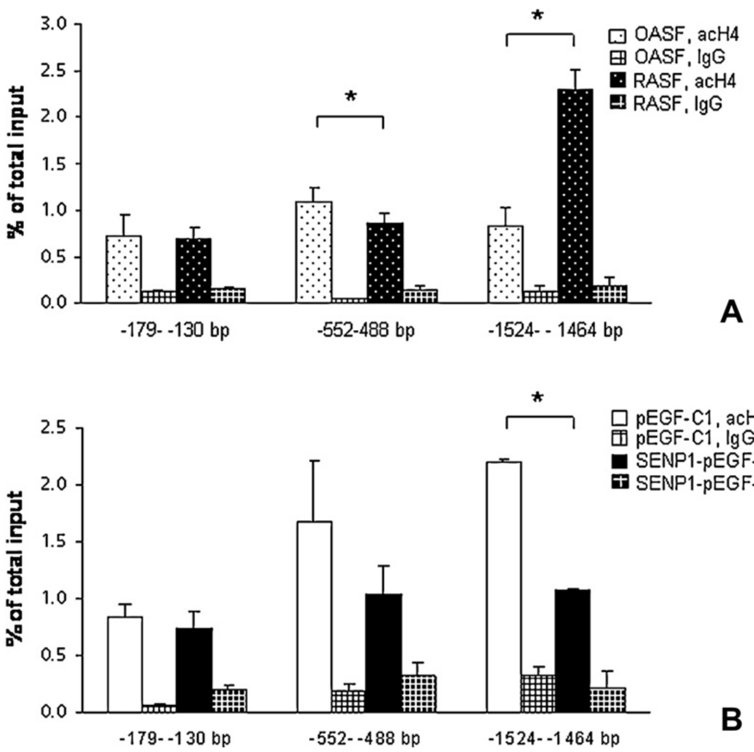

Fig. 1. Acetylation of histone H4 in the promoter of MMP-1. Chromatin immunoprecipitation assay showing acetylated histone H4 in the promoter regions of MMP-1 (−179 to −130 bp, −552 to −488 bp and −1524 to −1464 bp regions upstream from the transcription staring point). (A) Baseline levels of acetylated histone H4 in three regions of the MMP-1 promoter in RASF compared to OASF ($n = 3$ each). The levels of acetylated histone H4 are given in % of input ± SEM and shown in white and black bars respectively. IgG serves as a negative control (paned bars). (B) Levels of acetylated histone H4 in three regions of the MMP-1 promoter in RASF following the overexpression of SENP1. The levels of acetylated histone H4 in mock ($n = 3$) and SENP1 transfected RASF ($n = 3$) are given in % of input ± SEM and shown in white and black bars respectively. IgG serves as a negative control (paned bars).

analyze whether it resulted in a repression of MMP-1 transcription. Indeed, the overexpression of SENP1 downregulated the expression of MMP-1 mRNA by (mean ± SEM) 58 ± 7% ($n = 5$, $p \leq 0.05$, Fig. 3A). Furthermore, the protein levels of MMP-1 were significantly decreased by 28 ± 6% ($n = 3$, $p \leq 0.05$) in RASF overexpressing SENP1 compared to mock transfected cells (Fig. 3B).

3.5. Overexpression of SENP1 in RASF results in a functionally reduced invasiveness

Next, since we observed that the overexpression of SENP1 downregulated the expression of MMP-1 in RASF, we wanted to investigate whether therefore also the invasiveness of these cells decreased. Indeed, using an in vitro functional invasion assay, we could observe that RASF transfected with SENP1 for 23 h were significantly less invasive (2 ± 2% decrease in TEER) compared to the mock transfected cells (34 ± 9% decrease in TEER, $p \leq 0.05$, Fig. 3C and D). At the earlier time points (12 and 19 h after transfection) we could not observe a significant difference in the invasiveness of SENP1 overexpressing RASF compared to the control cells (Fig. 3D). In conclusion, we could show that the overexpression of SENP1 in RASF not only alters the expression of MMP-1 but also functionally interferes with the invasive phenotype of RASF.

3.6. Overexpression of SENP1 leads to an accumulation of histone deacetylase HDAC4 on the MMP-1 promoter

Since SENP1 overexpression results in a decrease in acetylation of histones at the MMP-1 promoter, we were interested whether this is due to the counterplaying histone deacetylases. Since HDAC4 is a substrate for sumoylation and this modification is connected with a change of localization of HDAC4, we hypothesised that the overexpression of SENP1 could mediate histone deacetylation through localizing HDAC4 to specific regions of chromatin. To investigate whether this is the case, we performed chromatin immunoprecipitation using anti-HDAC4 antibodies in RASF transfected with the SENP1 expression vector or with the mock control. Next, Real-time PCR for the different regions of the MMP-1 promoter was performed. The level of HDAC4 in the region at −1524 to −1464 bp from the transcription starting point was significantly increased in RASF overexpressing SENP1 when compared to the mock transfected cells by (mean ± SEM) 197 ± 36% ($n = 3$, $p \leq 0.05$, Fig. 4A). There was no significant change in HDAC4 detected in the two other analysed regions. Therefore, the overexpression of SENP1 resulted in a significant increase of HDAC4 localized to the MMP-1 promoter and to the significantly decreased acetylation of histone 4 in the same region of the promoter.

3.7. SENP1 requires HDAC4 for downregulation of MMP-1 expression

Since SENP1 overexpression lead to an increase in the localization of HDAC4 and at the same time to a decrease of histone acetylation in the promoter of MMP-1, we were interested whether HDAC4 mediates the downregulation of MMP-1 expression in SENP1 overexpressing cells. Therefore, we silenced the expression of HDAC4 and next overexpressed SENP1. As expected, SENP1

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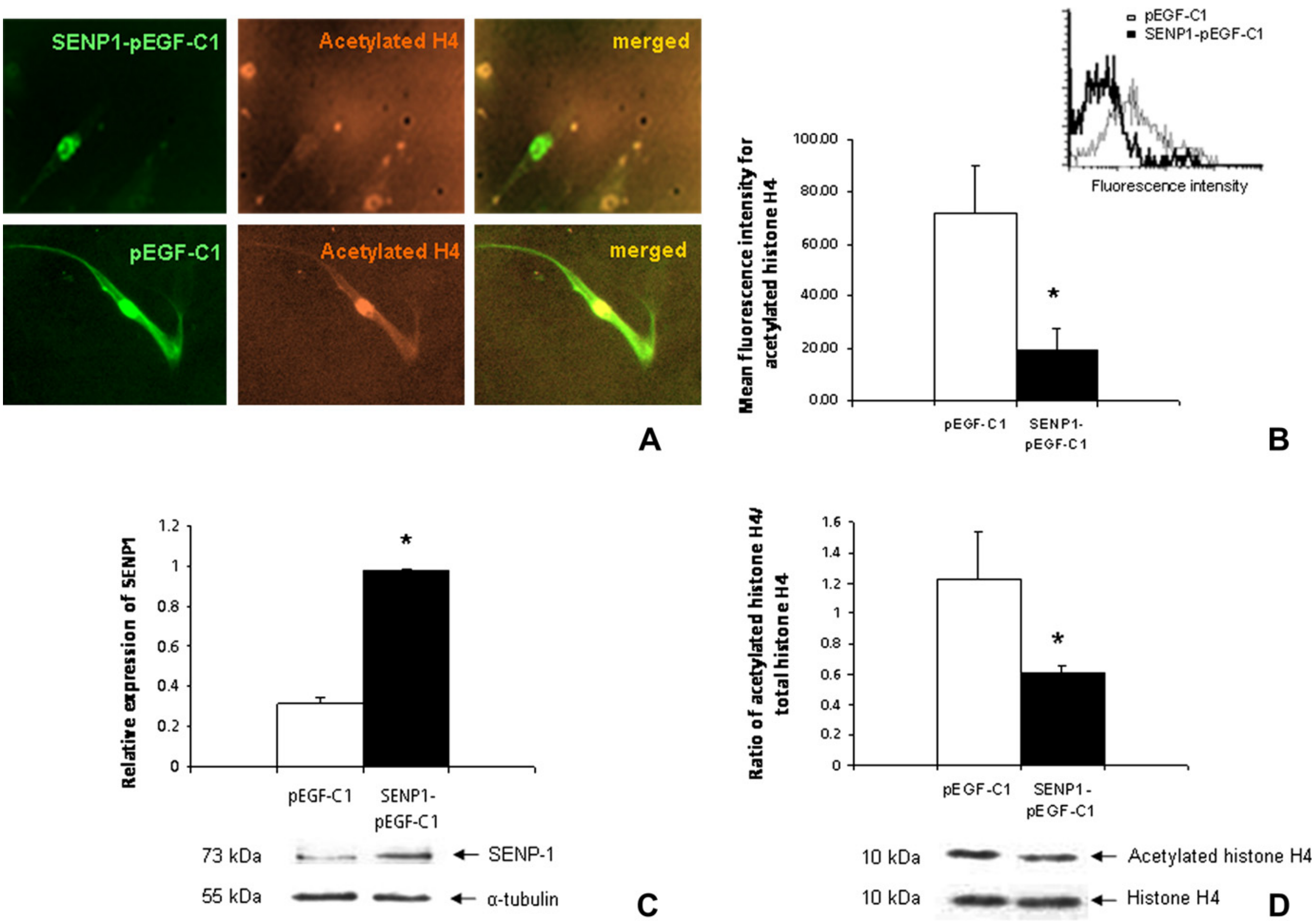

Fig. 2. Modulation of global acetylation of histone H4 in RASF by overexpression of SENP1. (A) Immunofluorescent staining of acetylated histone H4 (red) in RASF transfected with SENP1–pEGF-C1 (green, upper panel) or mock pEGF-C1 vector (green, lower panel) (B) Levels of acetylated histone H4 in control cells transfected with the empty vector pEGF-C1 ($n = 3$, white bar) and SENP1 transfected cells ($n = 3$, black bars) measured by FACS are shown as mean fluorescence intensity ± SEM. Representative FACS picture for acetylated histone H4 in SENP1–pEGF-C1 and pEGF-C1 transfected cells is shown in the inset. (C) Representative Western blot showing levels of SENP1 in mock-and SENP1-transfected RASF. As internal control α-tubuline was used. The levels of SENP1 expression in mock- (white bar) and SENP1-transfected RASF (black bar) were quantified using densitometric analysis software and normalized against α-tubulin. The values from 3 experiments are shown as mean ratio of densospot units ± SEM. (D) Representative Western blot showing levels of acetylated histone H4 in mock-and SENP1-transfected RASF (upper panel). As internal control histone H4 was used (lower panel). The ratio of acetylated histone H4/histone H4 in mock- (white bar) and SENP1-transfected RASF (black bar) were quantified using densitometric analysis software. The values from 3 experiments are shown as mean ratio of densospot units ± SEM.

overexpression in cells transfected with control siRNA lead to a decreased MMP-1 mRNA production. Most interestingly, in the cells silenced for HDAC4 and next transfected with SENP1 the levels of MMP-1 mRNA were significantly higher than in the cells transfected with SENP1 but expressing HDAC4 (percent change 190 ± SEM 20%, $n = 3$, $p \leq 0.05$, Fig. 4B). Also at the protein level, in the cells silenced for the expression of HDAC4 and next transfected with SENP1 we could not observe a downregulation of MMP-1 in contrast to the cells transfected with control siRNA and next SENP1-pEGF-C1 (percent difference 156 ± SEM 9%, Fig. 4C). Therefore, SENP1 requires HDAC4 for the downregulation of MMP-1 expression.

4. Discussion

We could show here for the first time that the MMP-1 promoter is intrinsically hyperacetylated in RASF when compared to OASF. Most interestingly, the increased cleavage of the intrinsically overexpressed SUMO-1 in RASF by SENP1 re-established the acetylation pattern in the MMP-1 promoter by deacetylation of histones in the distal fragment of the MMP-1 promoter. Furthermore, the overexpression of SENP1 led to a decrease in the production of MMP-1 and functionally interfered with the invasive phenotype of RASF. The transcriptional repression of MMP-1 was mediated by an epigenetic mechanism and depended on HDAC4.

It has been described by Huber et al. that in RA synovial tissues the balance of HAT/HDAC activity is strongly shifted towards histone acetylation [8]. In addition, it has been shown that several HDAC inhibitors had rather beneficial effects in the animal models, where they were shown to improve joint swelling, synovial inflammation, bone and cartilage destruction, downregulated the production of VEGF, blocked angiogenesis and promoted cell cycle arrest and apoptosis [25–28]. These reports suggest that HDACs could be misplaced in the subcellular compartments for example being localized aberrantly to specific promoter sites, and thereby contribute further to the pathogenesis of RA. Therefore, it should be carefully considered whether inhibition of HDACs or rather their redirection to a proper localization is more feasible for the treatment of RA.

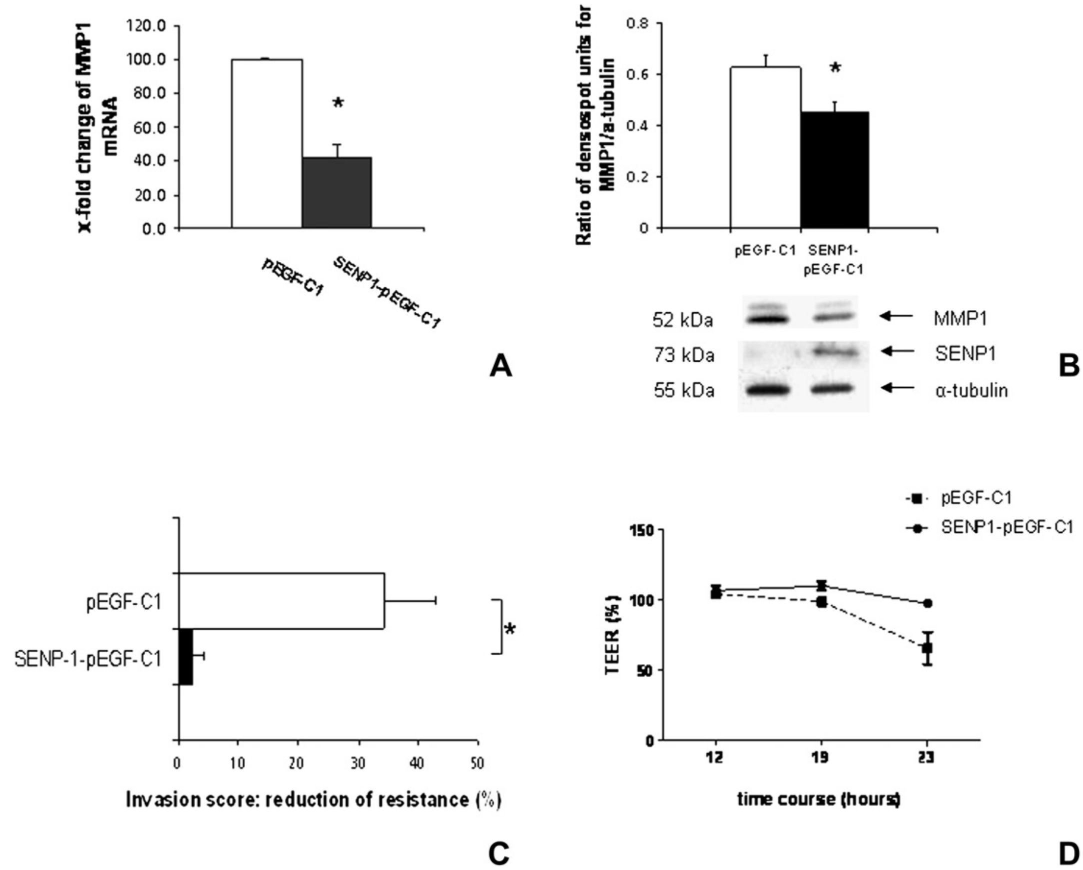

Fig. 3. Modulation of MMP-1 expression and invasiveness of RASF by overexpression of SENP1. (A) Levels of MMP-1 after transfection of RASF with SENP1. Levels of MMP-1 mRNA in the control cells transfected with the empty vector pEGF-C1 were set as 100% (white bar) and respective values for SENP1 transfected cells ($n = 5$, black bars) are shown as mean fold change ± SEM. Values were normalized for the expression of 18S. (B) Representative Western blot showing levels of MMP-1 expression in mock-and SENP1-transfected RASF (upper panel). SENP1 in mock-and SENP1 transfected cells is shown in the middle panel. As internal control α-tubuline was used. The levels of MMP-1 expression in mock- (white bar) and SENP1-transfected RASF (black bar) were quantified using densitometric analysis software and normalized against α-tubulin. The values from 3 experiments are shown as mean ratio of densospot units for MMP-1/α-tubulin change ± SEM. (C) Invasiveness of RASF transfected with mock vector pEGF-C1 (white bar) or SENP1 (black bar) shown as % reduction of electrical resistance of a monolayer of MDCK-C7 cells. (D) Invasiveness of RASF transfected with mock vector pEGF-C1 or SENP1 shown as % of electrical resistance of a monolayer of MDCK-C7 (TEER) cells at different time points, where the resistance at time 0 was set as 100%.

RASF play a crucial role in cartilage destruction by active invasion via producing MMPs [6]. Therefore, targeting of the intrinsically activated RASF could lead to the development of new therapeutic strategies for RA. We could previously show that RASF are characterised by high expression levels of SUMO-1 and at the same time low levels of SENP1 [12]. Here we demonstrate that reversing this balance by overexpression of SENP1 leads to a decrease in the invasiveness of RASF and downregulates the expression of MMP-1. Most interestingly, this regulation is dependent on a transcription inhibitor, HDAC4, since in cells silenced for HDAC4 the overexpression of SENP1 failed to decrease the expression of MMP-1. Furthermore, we could show that upon the overexpression of SENP1 in RASF, HDAC4, localized especially to the distal region of the MMP-1 promoter. HDAC4 has been shown to be modulated by SUMO-1 by a SUMO ligase RanBP2 in the nuclear pore complex [16]. This suggests that sumoylation of HDAC4 promotes nuclear transport of the modified protein. Such a mechanism has been shown for nuclear translocation of the insulin gene regulator Pdx1 as well as the tumor suppressor Smad4 [29–31]. We show here that SENP1 overexpression in the nucleus of RASF led further to the specific accumulation of HDAC4 on the DNA. Furthermore, SENP1 driven modulation of HDAC4 localization re-established the acetylation pattern in the MMP-1 promoter in RASF by deacetylation of histones in the distal fragment of the MMP-1 promoter. This promoter region contains predicted binding sites for acetyltransferases such as C/EBPα and p300. Moreover, this promoter region contains a predicted binding site for IRF-3, which was described to be superactivated in RA synovial tissues [32], to be regulated by HDAC inhibitors [33] and to interact with PIASy, a SUMO ligase [34]. It needs to be investigated whether IRF-3 regulates the transcription of MMP-1. On the contrary, no significant changes were observed in both acetylation of histones and HDAC4 levels after the overexpression of SENP1 in the AP-1 binding site proximal to the transcription starting point (−179 to −130 upstream from the transcription starting point) as well as the region −552 to −488 upstream from the transcription starting point to which no histone acetyltransferases have been predicted to bind.

Most interestingly, we were able to show that the overexpression of SENP1 leads to a decrease in the global acetylation of chromatin. This is the first study showing that interference with the sumoylation pathway can decrease the invasiveness of RASF and down-modulates the expression of MMP-1 via epigenetic changes in the chromatin. We therefore propose that the sumoylation pathway could be a promising target for therapy in RA, by inhibiting both the resistance to induced apoptosis and the invasiveness of RASF mediated by MMP-1.

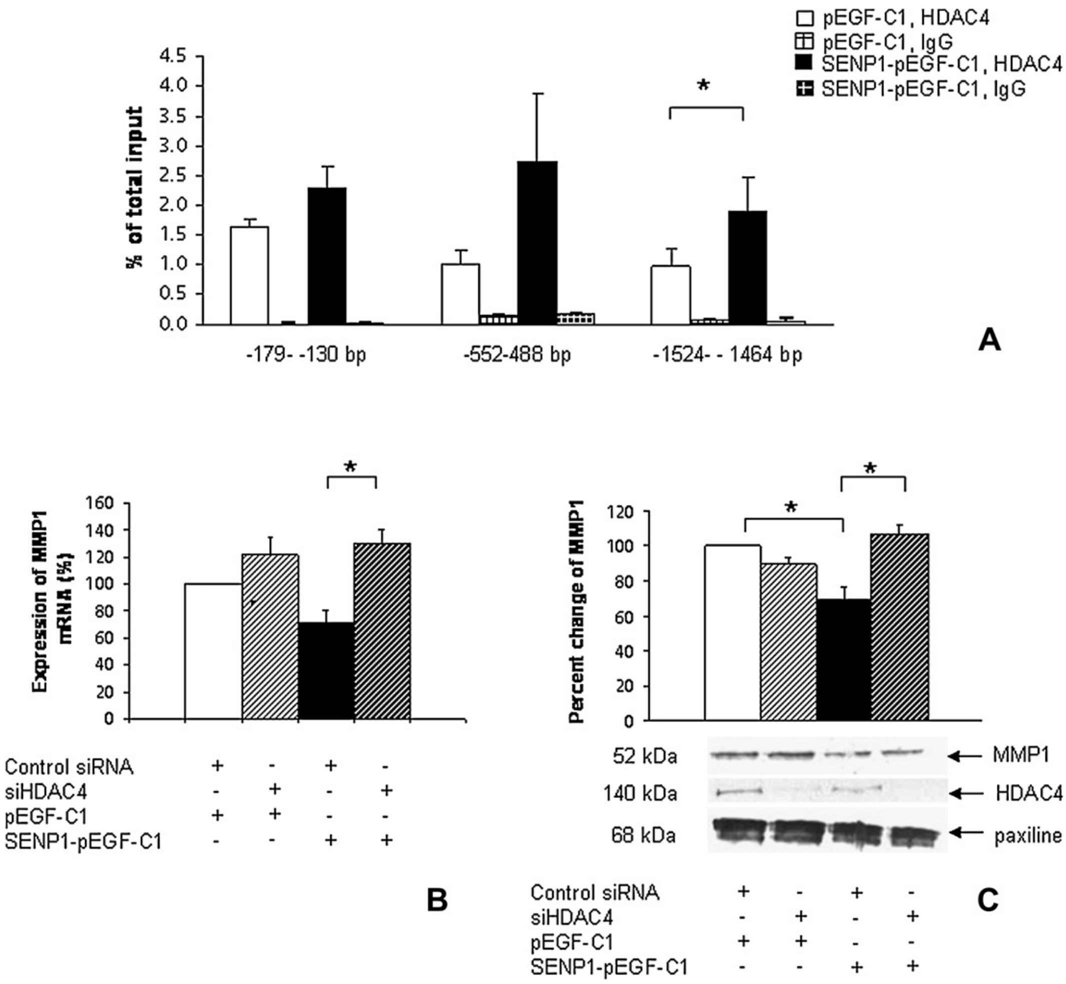

Fig. 4. HDAC4 driven downregulation of MMP-1 expression in RASF overexpressing SENP1. (A) Levels of HDAC4 in three regions of the MMP-1 promoter in RASF overexpressing SENP1. The levels of HDAC4 in mock ($n = 3$, white bar) and SENP1 transfected RASF ($n = 3$, black bar) are given as % of input ± SEM. IgG serves as a negative control (paned bars). (B) Levels of MMP-1 mRNA in RASF measured by Taqman. Levels of MMP-1 mRNA in control cells transfected with siRNA/pEGF-C1 were set as 100% ($n = 3$, white bar). Relative values of MMP-1 mRNA levels are shown as mean fold change ± SEM for cells transfected with siHDAC4/pEGF-C1 ($n = 3$, white striped bar), control siRNA/SENP1-pEGF-C1 ($n = 3$, black bar) and siHDAC4/SENP1 ($n = 3$, black striped bar). (C) Levels of MMP-1 in the cytoplasmic fraction of RASF transfected with a control siRNA/pEGF-C1 ($n = 3$, white bar), siHDAC4/pEGF-C1 ($n = 3$, white striped bar), control siRNA/SENP1 ($n = 3$, black bar) and siHDAC4/SENP1 ($n = 3$, black striped bar) measured with densitometric analysis software. The values for MMP-1 were normalized for the expression of cytoplasmic protein paxillin. The levels of MMP-1 in control siRNA/pEGF-C1 transfected cells were set as 100%. Values are given as fold change ± SEM. Representative Western blot is shown ($n = 3$).

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4 Outlook

4.1 Autoantibodies

4.1.1 Anti-serpin E2 autoantibodies

In the first part of my PhD work I identified novel and functional autoantibodies against serpin E2 in sera and synovial fluids of RA patients. The analysis of a bigger cohort of sera which was performed in a follow up study in our laboratory showed that the anti-serpin E2 autoantibodies are also present at high levels in sera of healthy individuals. Since I could show that anti-serpin E2 autoantibodies can abbrogate serpin E2 inhibitory activity towards uPA, most probably they have a functional role in the physiological conditions. It would be interesting to analyze whether healthy individuals with high and low serum levels of anti-serpin E2 differ regarding for example predisposition to develop thrombosis, arthritis or cancer. Furthermore, by immunohistochemistry, I could show that serpin E2 is overexpressed in the synovial tissues of RA patients. It remains unresolved what is the level of serpin E2 activity in the synovium of RA patients. Even though serpin E2 is overexpressed, it might be inhibited by the anti-serpin E2 autoantibodies, which are present at increased levels in the synovial fluids of patients with RA when compared to OA. It also remains to be verified whether the increased levels of serpin E2 drive the retention of the leukocytes specific for serpin E2 and/or their activation and therefore the increased production of serpin E2 specific autoantibodies. Otherwise, the increased presence of anti-serpin E2 autoantibodies as a negative regulator of serpin E2 could upregulate the expression of the latter. Whether the binding of the specific autoantibodies to serpin E2 can drive the expression of the antigen could be verified in vitro for example by incubating fibroblasts with the isolated anti-serpin E2 autoantibodies alone or together with serpin E2. It has been reported that recombinant human serpin E2 administrated in a rabbit model of arthritis improved the symptoms [174]. To study the inhibitory effects of anti-serpin E2 autoantibodies in an in vivo model, it would be interesting to verify if administration of serpin E2 along with anti-serpin E2 autoantibodies isolated from sera had similar effects. In the human circulation serpin E2 is expressed by platelets [175]. Platelet serpin E2 was shown to have antithrombotic properties since it inhibits thrombin and is thought to be a key player in the thrombotic process [175]. In this respect, it is possible that anti-serpin E2 autoantibodies could play a role as a prothrombotic regulator. While in the PhD work I have shown that the levels of of anti-serpin E2 autoantibodies correlate with the activity of another protease inhibited by serpin E2, namely uPA, whether anti-serpin E2 autoantibodies correlate with the activity of thrombin remains to be verified. A similar approach to the one where the inhibition of uPA was studied could be used in the case of thrombin. On the other hand, in the pathologic conditions, serpin E2 was found to be overexpressed in atherothrombotic lesions [175]. It is perhaps not surprising that at the site of increased activity of thrombin its negative regulator is present. Nevertheless, whether serpin E2 at this site is bound and inhibited by the naturally occurring autoantibodies remains unverified. If so, even the strong expression of serpin E2 observed in advanced lesions could be insufficient to inhibit thrombin. In this view a naturally occurring anti-serpin E2 autoantibodies could play a pathological role at the site of atherothrombotic lesions. It would be therefore interesting to verify whether the anti-serpin E2 autoantibodies are present at the site of the atherothrombotic lesions and if they correlate with the activity of thrombin and/or the size of the atherothrombotic plaque.

4.1.2 Other novel autoantibodies

Except from serpin E2, using the SEREX technique I identified four other potential autoantigens, namely: CTGF, GLOI, NRG1 and PTTG1IP. It needs further investigation whether there are autoantibodies against these molecules present in more individuals, and whether they are connected with RA. The potential autoreactivity of CTGF is of interest since CTGF is a key player in another autoimmune disease, systemic sclerosis (SSc). SSc is an autoimmune disorder in which pathologic features include progressive tissue fibrosis and widespread vascular involvement. CTGF is overexpressed in SSc and is believed to be a primary mediator of chronic fibrosis [176, 177]. It would be therefore interesting to verify the existence of anti-CTGF autoantibodies not only in RA, but also in the circulation of patients with SSc as well as other autoimmune diseases. Another interesting possible autoantigen identified in the study was glyoxalase I (GLOI). GLOI is an enzyme involved in the detoxification of a metabolic by-product, methylglyoxal. Targeting GLOI appears promising in the treatment for cancer since the methylglyoxal is preferentially toxic to the cells proliferating at a high rate [178, 179]. Little however is known about GLOI in RA, except that GLOI mRNA is increased twice in RA compared to OA tissues [180]. It would be therefore interesting to verify the autoantibodies against GLOI in RA. It might be of interest whether possible anti-GLOI autoantibodies could interfere with the activity of GLOI in a similar manner like anti-serpin E2 autoantibodies block their antigen activity. If anti-GLOI antibodies indeed had blocking properties, it could be hypothesized that they could play a role in the regulation of cell proliferation. Furthermore, such an inhibitor could be of potential value as an inhibitor of cancer development. It could be therefore interesting to study whether there exists any relation between the presence of anti-GLOI antibodies and the risk of developing cancer. A next potential novel autoantibodies identified in my study is anti-NRG1. Interestingly, NRG1 has an Ig-like domain. It is intriguing whether autoimmunity could rise to such a protein especially in the RF positive patients due to the recognition of the Ig-like domain by the RF. On the other hand, the identification of NRG1 as a potential autoantigen using the SEREX technique might be simply an artifact, since it is possible that the anti-IgG antibodies used for detection of the autoantibodies bound directly the Ig-like domain of NRG1. Due to the same reason, even if there exist anti-NRG1 autoantibodies, their further study would be very challenging. Pituitary tumor-transforming gene 1 (PTTG1) interacting protein (PTTG1IP) was another possible autoantigen identified using the SEREX technique. PTTG1IP was shown to have both pro- and anti-tumorigenic properties [181]. Up to date there are no reports linking PTTG1IP to autoimmunity. Nevertheless, PTTG1IP has been suggested to play a role in osteoblast differentiation, a process not efficient in RA [182]. It would be therefore interesting to investigate the expression and function of PTTG1IP in cartilage and synovium in the RA joint. It is possible that PTTG1IP could increase the invasiveness of the resident cells in the synovial tissue since it was reported to play such a role in breast cancer cells. Furthermore, since PTTG1IP has been suggested to play a role in osteoblast differentiation it might be interesting to verify whether the autoantibodies against PTTG1IP interfere with osteoblast differentiation and attempted repair processes in the affected joints in RA.

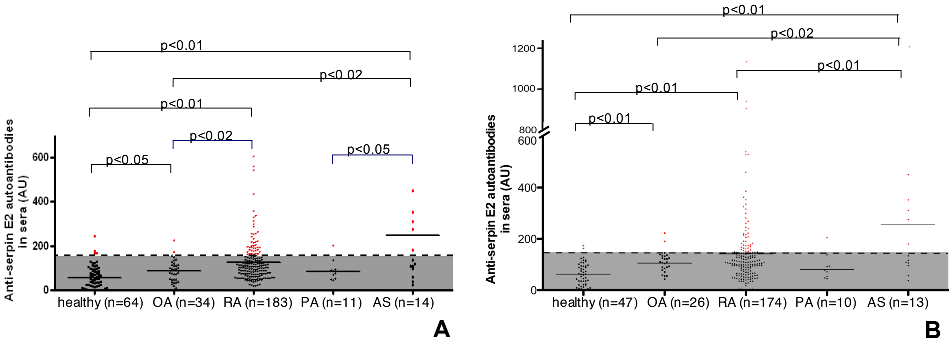
4.2 SENP1 study

In the second part of my PhD work I showed that the expression of MMP-1 is regulated by SENP1. I showed that SENP1 overexpression leads to the localization of HDAC4 and deacetylation of histone H4 in the promoter of MMP-1. Even though I could show that in the cells silenced for HDAC4 SENP1 overexpression failed to downregulate MMP-1, it could be also investigated whether in those cells the acetylation of MMP-1 promoter remains unchanged upon SENP1 overexpression. Even though it is clear that SENP1 requires HDAC4 for regulating expression of MMP-1, the exact mechanism remains unknown. It is of high interest to provide such a mechanism since the inhibition of HDACs proved beneficial in several animal models of RA [34, 35] even though in RA synovial tissues the balance of HAT/HDAC activity is strongly shifted towards histone acetylation [33]. This discrepancy could be explained if HDACs were localized aberrantly to specific promoter sites, and thereby contributed further to the pathogenesis of RA. If that was the case, the possibility of redirection of HDACs to proper sites would be very desired. Since sumoylation of HDAC4 can affect its localization it is possible that SENP1 driven desumoylation of HDAC4 would change the location of HDAC4. This could happen on the cytoplasm-nucleus or intranuclear level and, as a result, HDAC4 could be recruited to particular promoter sites. The more probable of this two hypothesis is the first one since the deacetylation of histone H4 driven by SENP1 overexpression was observed not only on the promoter of MMP-1 but also globally. It could be addressed by Western blot analysis of HDAC4 in the cytoplasmic and nuclear fractions in the cells transfected with SENP1. The intranuclear changes in the localization of HDAC4 could be studied using Chip on Chip technique. On the other hand, SENP1 overexpression could lead to an increase in the total levels of HDAC4. This could be accomplished for example via SP1/SP3 transcription factors, since they are described stimulators of HDAC4 transcription and they are active in their desumoylated form [183, 184, 185]. An increase in HDAC4 expression could result in its increased binding to the promoter of MMP-1 while a decrease in the total acetylated histone H4 might be observed. It is also possible that sumoylation/desumoylation of HDAC4 influences its interactions with transctription regulators. It would be therefore interesting to compare the transcription factors interacting with the wild type and the sumoylation mutant of HDAC4. This could be addressed using the yeast two hybrid system. Another interesting point worth of studying in the future is the novel regulatory region identified in the promoter of MMP-1. The acetylation of histones in the region -1524 - -1464 upstream from the transcription site was different in RASF and OASF and a decrease in the acetylation of H4 in this region was paralleled by decreased levels of MMP1 expression. This promoter region was also identified as HDAC4 binding site. Also other transcription factors are predicted to bind to this region (STAT5A, C/EBPα, p300, GATA1, IRF-3, MZF-1, BTEB3). It would be interesting to study this promoter region in more detail, for example if it is necessary for the upregulation of MMP-1 by proinflamatory cytokines such as TNFα. It has recently been reported that HDAC4 regulates the expression of another collagenase, MMP-13 [186]. It might be suggested that SENP1 downregulated also MMP-13 (via HDAC4), since the overexpression of SENP1 decreased the invasiveness of RASF much stronger then expected given the 30% change in the expression of MMP-1. Furthermore, it could be investigated whether other classes of MMPs are also regulated in a similar manner.

Corrigendum

The following corrigendum to the article entitled 'Functional autoantibodies against serpin E2 in rheumatoid arthritis characterizing novel anti-serpin E2 autoantibodies present in RA' Arthritis Rheum. 2010 Jan;62(1):93-104 will be sent together with an addendum to the journal.

We published an article in Arthritis Rheum. 2010 Jan;62(1):93-104. entitled Functional autoantibodies against serpin E2 in rheumatoid arthritis characterizing novel anti-serpin E2 autoantibodies present in RA. We regret that in the Figure 3B a wrong data set for the concentrations of anti-serpine2 autoantibodies in sera was presented. The corected data are shown in Figure 1 corrigendum. We apologize for any problems arising from this error.



	Mean anti-serpin E2 (AU)		% positive	
diagnosis	published	**correct**	published	**correct**
healthy	61	**63**	6	**4**
OA	86	**105**	6	**8**
RA	125	**142**	22	**25**
PA	81	**81**	9	**10**
AS	247	**264**	33	**46**

C

Figure 1 corrigendum : Comparison of published vs. corrected values of the levels of anti-serpin E2 autoantibodies in sera. Levels of autoantibodies reactive with rh serpin E2 in sera published as Figure 3A in the manuscript (A) and corrected (B). Dots represent values obtained for each individual and the mean of total samples in each group is indicated with a black bar. The red dots represent positive individuals. P values were calculated using the Student T Test and p≤0.05 considered significant are indicated. The published and the corrected values are summarized in a table (C). The mean levels of anti-serpin E2 autoantibodies detected by ELISA in sera are presented in the left panel. The percentage of sera positive for anti-serpin E2 autoantibodies is given in the right panel. (AU = arbitrary units). The values published in the manuscript are presented in the first column and the corrected values in bold in the second column in both pannels.

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Abbreviations

ACR	American College of Rheumatology
ALDOA	aldolase A
anti-CCP	anti-cyclic citrullinated protein antibodies
AS	ankylosing spondylitis
CC	chemokine
CCL	chemokine ligand
cDNA	complementary DNA
CIA	collagen induced arthritis
CpG	deoxy-cytidylate-phosphate-deoxy-guanylate
Ct	threshold cycle
CTGF	connective tissue growth factor
DC	dendritic cell
DCN	decorin
DISC	death inducing signaling complex
DMEM	dulbeccos modified eagle medium
DMARDs	disease-modifying antirheumatic drugs
DNA	deoxyribonucleic acid
dNTP	deoxynucleotriphosphate
ECM	extracellular matrix
EEF1A1	elongation factor 1-alpha 1
ELISA	enzyme-linked immunosorbent assay
FACS	flow cytometry analysis
FADD	Fas-associated via death domain
FCS	fetal calf serum
GFP	green fluorescent protein
GLOI	glyoxalase I

HAT	histone acetyltransferase
HDAC	histone deacetylase
HLA	human histocompatibility leukocyte antigen
HRP	horseradish peroxidase
IFN	interferone
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl β-D-1-thiogalactopyranoside
IRF	interferon regulatory factor
LINE-1	long interspersed nuclear element 1
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
MATRIN	matrix-associated transepithelial resistance invasion assay
miRNA	micro RNA
MMP	matrix metalloproteinase
mRNA	messenger RNA
NBT	nitro blue tetrazolium
NF-kB	nuclear factor kappa B
NO	nitric oxide
NOR	nucleolar organizer regions
NRG1	neuregulin 1
NRT	not reverse transcribed
NSAID	nonsteroidal anti-inflammatory drugs
OA	osteoarthritis
OASF	OA synovial fibroblast
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor 1
PAMP	pathogen associated molecular pattern
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PML	promyelocytic leukemia
PRR	pattern recognition receptor
PsA	psoriatic arthritis

PTTG1IP	pituitary tumor-transforming gene 1 protein-interacting protein
RA	rheumatoid arthritis
RANK	receptor activator of NF-kB
RANKL	RANK ligand
RASF	RA synovial fibroblast
RCL	reactive center loop
RF	rheumatoid factor
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription PCR
SCID	severe combined immunodeficiency
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SENP1	sentrin/sumo specific protease
SEREX	serological analysis of cDNA expression library
siRNA	small interfering RNA
Serpin E2	serine protease inhibitor E2
SUMO-1	small ubiquitin-like modifier
TGFβ	transforming growth factor β
Th	T helper cell
TIMP	tissue inhibitor of metalloproteinases
TLR	toll-like receptor
TNFα	tumor necrosis factor α
TNFRI	TNF receptor I
tPA	tissue plasminogen activator
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory cell
TSA	trichostatin A
uPA	urokinase-type plasminogen activator
uPAR	uPA receptor
VIM	vimentin

Curriculum vitae

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